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Characterization of mouse models to study the pathogenesis of celiac disease and the role played by the dysregulation of the intestinal microbiota.

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ABSTRACT

Celiac disease (CD) is a permanent intolerance to dietary protein, gluten, from wheat rye and barley. It occurs in about 1% worldwide population, in genetically predisposed individuals bearing human leukocyte antigen (HLA) DQ2/DQ8. Although gut epithelial cell stress and the innate immune activation are responsible for the breaking oral tolerance to gliadin, the gluten component, the exact mechanisms through which gliadin can stimulate CD onset are still unclear. Here I show how is important to identify *in vivo* preclinical models of CD to study its pathogenesis, at molecular level.

The increasing prevalence of positive serological marker of CD in Cystic Fibrosis (CF) affected patients let to the hypothesis of a link between the two disorders. Results from my studies indicate that CFTR is potentially involved in the pathogenesis of CD, with gliadin peptides inhibiting CFTR activity and expression.

To date, the only treatment available for CD is a long-term gluten-free diet. Several evidences show that an altered composition of the intestinal microbiota (dysbiosis) could play a key role in the pathogenesis of CD, through the modulation of intestinal permeability and the regulation of the immune system. Indeed, although further studies are still required to unveil the molecular mechanisms, results reported in the present work clearly indicate that rebalancing the gut microbiota composition by probiotics administration might represent a new strategy to treat CD affected patients.

INTRODUCTION

Celiac Disease is a complex immune-mediated chronic disease characterized by strong inflammatory reactions to dietary gluten intake by genetically predisposed individuals, affecting the intestinal barrier.

1. THE INTESTINAL BARRIER

The intestinal mucosal barrier, also known as intestinal barrier, has a crucial role in the defence from pathogens and from potentially harmful substances present in the environment. The intestinal barrier is composed by physical, biochemical, and immune elements highly organized by the intestinal mucosa.

1.1 STRUCTURE AND FUNCTIONS

The central component of the intestinal barrier is represented by the epithelial layer, which provides physical separation between the lumen and the body (figure 1). The secretion of several molecules in the lumen help the barrier function on the extraepithelial side, and a diversity of immune cells provide protection below the epithelial layer. Immune cells, intestinal microbiota and anti-microbial peptides have the main role in maintaining the intestinal barrier function (Takiishi et al., 2017). In the gastrointestinal tract (GI), the mucus barrier forms a protective layer on the apical surface of the intestinal epithelium to avoid adherence and subsequent invasion by external pathogens. Moreover, the mucus lubricates food and digestive secretion, protecting the intestinal epithelium from potential damages (Johansson et al., 2013; Pelaseyed et al., 2014)

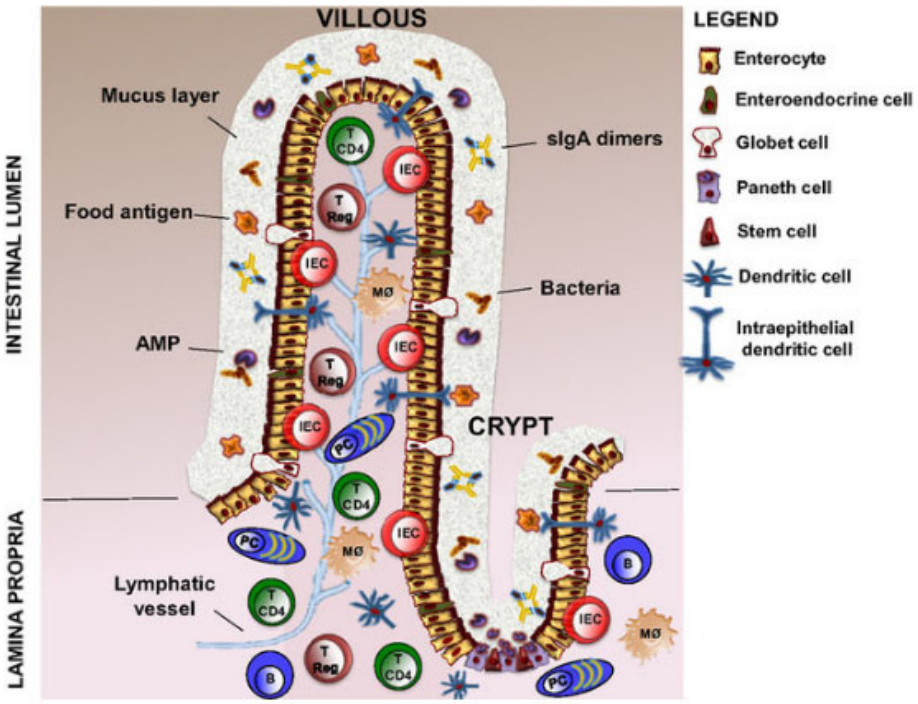

Figure 1. Structure of the intestinal barrier. *The intestinal barrier is made of cellular and extracellular elements. The cellular fraction is composed by intestinal epithelium and the underlying lamina propria, which contains DCs, macrophages, intraepithelial lymphocytes (IEL), T regulatory cells (T Regs), TCD4+ lymphocytes (T CD4), B lymphocytes, and plasma cells. The extracellular part is made of a mucus layer produced by Goblet cells, AMPs secreted by Paneth cells, and sIgA dimers released by plasma cells. DCs, dendritic cells; AMPs, antimicrobial peptides; sIgA, secretory Immunoglobulin A.* (De Santis et al., 2015)

1.2 MUCUS

Mucus forms a layer, or layers in the colon, gather the epithelium of the GI. It prevents large particles from contacting the epithelial cell but allowing the passage of small molecules. The mucus makes easier the passage of the luminal contents along the length of the gut, protects the epithelial cells from digestive enzymes, and prevents the direct contact between microorganisms and epithelial cells. Mucus is secreted by goblet cells and is formed of approximately 95% water. One of the mainly components of the mucus are mucins, which are glycosylated big proteins rich in serine and threonine (Bansil & Turner, 2006).

Complementary to that, lysozyme digest bacterial cell wall components (Nakimbugwe et al., 2006; Ragland & Criss, 2017). Immunoglobulins, specifically secreted IgA, contribute to mucosa homeostasis since lack of IgG leads to protein losing-enteropathy (Hooper et al., 2012). Grow factors such as transforming growth factor beta, are involved in growth, maintenance, repair and regulatory function in the epithelium (Scherf et al., 2005).

1.3 THE INTESTINAL EPITHELIUM

The epithelium covering of the intestinal wall represents the most important component of the intestinal barrier. Crucial for having a real barrier is the control of the paracellular pathway. Indeed, in addition to its protective function, the intestinal epithelium also controls the selective uptake of beneficial ions, nutrients, and other substances from the lumen.

In this single cell layer, cells are attached to each other by the apical junctional complex which seals the paracellular space to the intestinal lumen. Stem cells present in the crypts are responsible for the constant renewal of this epithelium, through cell division, maturation and cell migration.

The predominant cells in the small intestine, are enterocytes, devoted to the absorption of nutrients, while goblet cells are the main mucus-secreting cells, and with Paneth cells playing a crucial role in the host defense against bacteria and in the regulation of the microbiota, mainly though the production and release of defensins, members of a large family of cationic antimicrobial peptides representing an essential element of innate immunity (Pasupuleti et al., 2012). M cells are a subset of epithelial cells highly specialized for antigen sampling. In fact, they transport antigens and intact microorganisms from the gut lumen to the lamina propria, in order to present them to immune cells and thus igniting the immune response (Kucharzik et al., 2000). Finally, Tuft cells monitor the intestinal lumen, and in case of local injury or bacterial infection, they transmit signals to immune cells in the underlying epithelia, activating the immune response (Ting & von Moltke, 2019).

The ‘selective barrier’ activity carried out by intestinal epithelium rely on two major pathways: the transcellular and paracellular pathways. The former is involved in the transport and absorption of nutrients including amino acids, small peptides, fatty acids, vitamins, short-chain fatty acids (SCFA) and sugars, through epithelial cells (Den Besten et al., 2013).This is generally regulated by selective transporters and channels located on the apical and basolateral surface of epithelial cells. However, larger antigens (>600Da), such as dietary peptides, can also cross the barrier through the transcellular transport, mediated by enterocytes and consisting in coupled endocytosis (on the luminal side) and exocytosis (on the body side). Released antigens can then interact with immune cells (Ménard et al., 2010). Importantly, this process is thought to be critical for the oral tolerance (Pabst & Mowat, 2012). Larger antigenic molecules, such as microbial components, may also cross the epithelial barrier through M cells, which are located in the epithelium of Peyer’s patches or isolated lymphoid follicles (ILF) (Pabst & Mowat, 2012). Therefore, the uptake of luminal antigens through M cells and absorptive enterocytes plays a key role in maintaining the gut homeostasis.

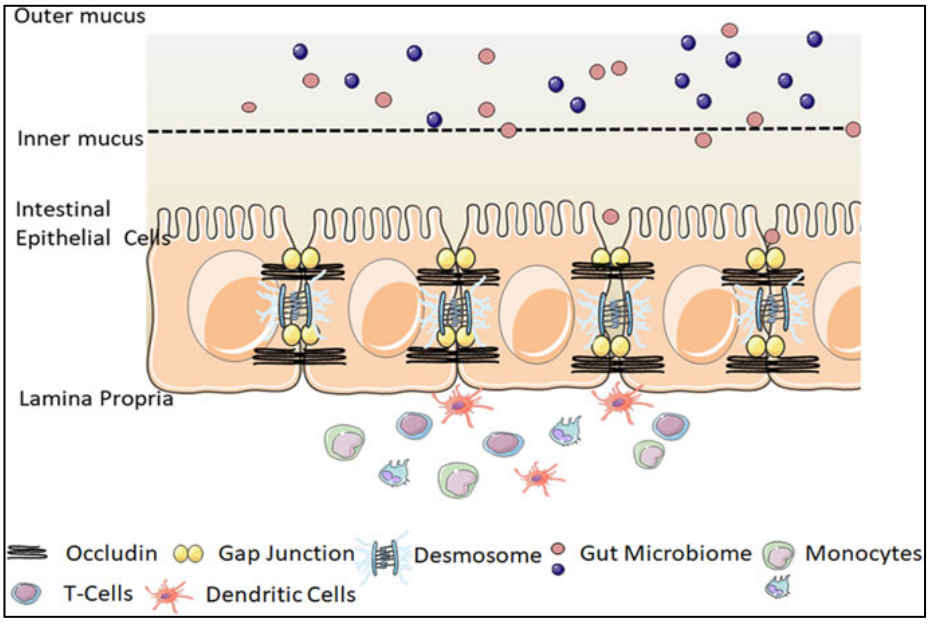

Figure 2. Intestinal epithelial barrier. *(Chaithanya et all 2018)*

1.4 INTESTINAL CELL JUNCTIONS

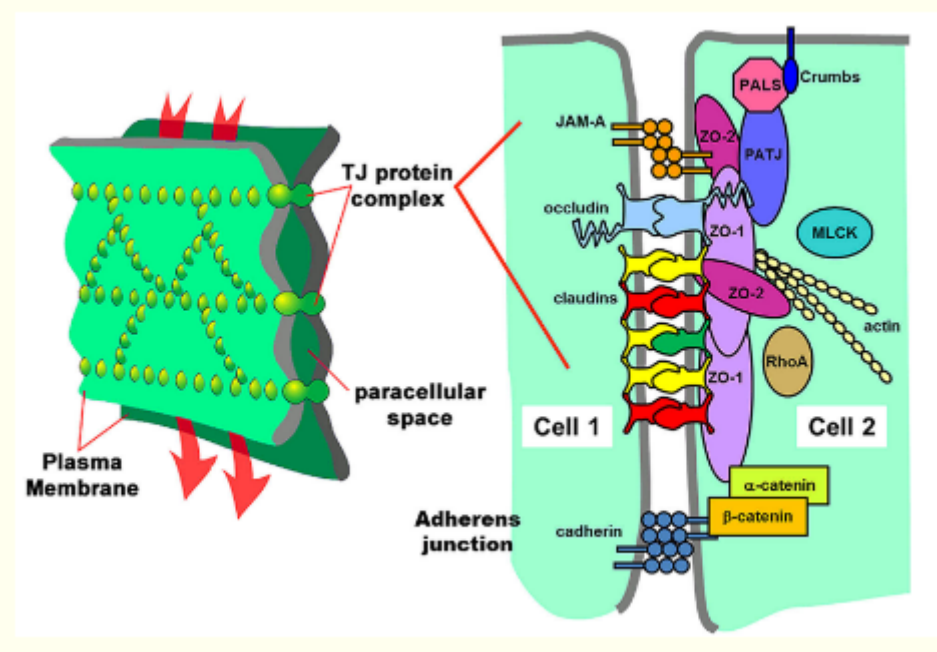

Figure3. TJ structure. *Tight junctions are the mainly mechanism which regulates whether the epithelium is tight or leaky. Tight junction permeability is primarily regulated by proteins in the claudin family that form a seal to both restrict paracellular diffusion and permit specific transport of ions between cells across the epithelial barrier.*

The barrier function of the intestinal epithelium mainly relays on the presence of specific cell-cell junctions occurring among epithelial cells, such as tight (TJ) and adherens junctions (AJ) (figure 3). TJs are located to the most apical part of the lateral epithelial cell membrane, which main functions were described as being a gate and a fence. In fact, they have the ability of selectively control the luminal components crossing the interepithelial space (gate function), and also to restrict lateral diffusion of membrane proteins as well as membrane lipids to either the apical or the basolateral compartment (fence). Main constituents of TJs include the transmembrane proteins occludins, the family of claudins and junctional adhesion molecules (JAM) (Citi, 2019). Although occludins were the first TJ component to be identified, their role remains somehow obscure, although they were reported to be involved in the regulation of paracellular permeability. On the contrary, claudins seem to be

the crucial component of TJ assuring the barrier function. Claudins also have functions in cytoskeleton organization, transport of vesicles and signaling pathways directly associated with scaffold proteins such as ZO-1 (Garcia-Hernandez et al., 2017). Alteration of the intestinal homeostasis is related to alterations in the expression of claudins.

Claudin dysfunction may contribute to epithelial permeability disorder and more intestinal diseases. Indeed, over recent years, the importance of claudins in the pathogenesis of inflammatory bowel diseases (IBD) has gained focus and is being investigated. Proinflammatory cytokines-induced small molecule (e.g., ions and mannitol) channel disruption and cell detachment-induced large molecule (e.g., epidermal growth factor, EGF) leakage (DiGuilio et al., 2016). Thus, as barrier-forming proteins, dysregulated expression and redistribution of claudins may lead to increased intestinal permeability, susceptibility to gut infection and bowel symptoms of IBD patients (Chang et al., 2017).

1.5 INTESTINAL IMMUNE SYSTEM

The gut immune system is characterized by high plasticity, being highly and promptly reactive toward pathogens, while maintaining tolerance toward innocuous antigens, including dietary antigens and commensal bacteria. Antigen-specific immune responses are generated at sites of induction, including mesenteric lymph nodes (MLNs), Peyer's patches, and ILF. Peyer's patches are located in the submucosa of the small intestine, while ILF are found in the lamina propria of the small and large intestine. The lamina propria and epithelium make up the effector sites and here there is a large population of lymphoid cells (Pabst & Mowat, 2012). Specialized M cells are located in the epithelium overlaying Peyer's patches, and are able to uptake the antigens and continuous sampling of luminal contents (Kobayashi et al., 2019). Antigens are then taken up by underlying antigen presenting cells (APCs), such as dendritic cells (DCs), which undergo maturation and present antigens to naïve $CD4^+$ T cells, thus activating the antigen-specific T helper (figure 4). Interactions

between CD4+ T cells and B cells in the Peyer's patches and MLN also results in the activation and expansion of B cells to become antibody-producing plasma cells. The local cytokine environment plays a key role in shaping the immune responses (figure 4). Following activation, $CD4^+$ T cells and B cells move back to the lamina propria as effector cells or antibody-producing plasma cells. Other immune cells and phagocytes, such as granulocytes, are also found in the intestinal lamina propria. These cells, especially migratory DCs and macrophages, play an important role in the adaptative immune response. Other important immune cell are the intraepithelial lymphocytes (IELs), which are specialized effector T cells residing within the epithelial barrier. IELs are the first line of defense for invading pathogen, are also involved in maintaining barrier function, due to their direct contact with IECs and close proximity to luminal antigens (Van Wijk & Cheroutre, 2010).This allows them to respond quickly to stress signals, thus representing an innate-like population of T cells (Gaudino & Kumar, 2019). IELs show cytolytic functions and are capable of producing pro-inflammatory cytokines in response to invading pathogens (figure 4). However, IELs also have regulatory functions that promote barrier function and epithelial repair after injury.

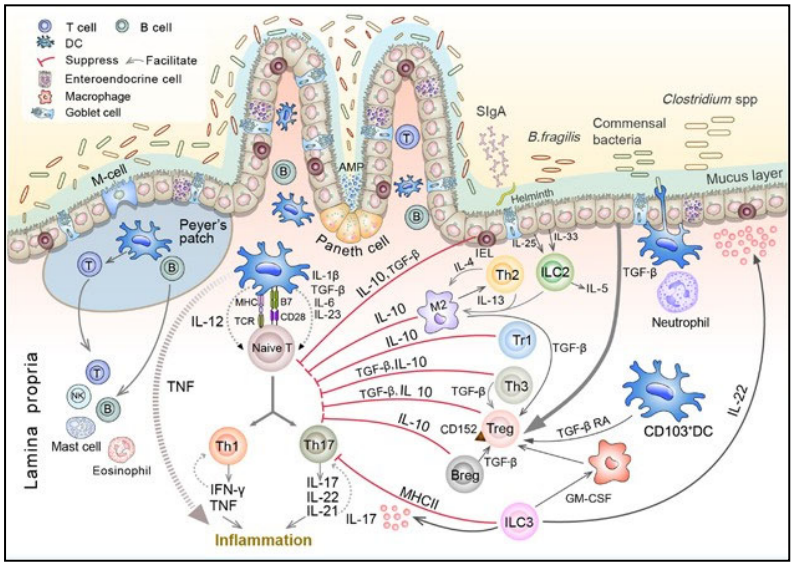

Figure 4. Immune system in the lamina propria of gut. *Intestinal epithelial barrier is the first line of defense to prevent the pathogens invasion, including the epithelial TJ complexes, mucus layer, secretory immunoglobulin A, antimicrobial protein secretion by plasma cells, and Paneth cells. With regard to the innate immune response in intestine, neutrophils could be earlier recruited to inflammatory sites to clear the pathogens. Moreover, macrophages, dendritic cells (DCs), and innate lymphoid cells (ILCs) could sense antigens and then secrete cytokines or chemokines to regulate the inflammatory responses (adapted by M. Sun et al 2015).*

2. CELIAC DISEASE

The intestinal immune system faces the lifelong challenge of identifying what is safe and what is not. Under physiological conditions, an efficient system guarantees correct tissue homeostasis and the capacity of suppressing inflammation and promoting oral tolerance to non-self-antigens such as those from diet or commensal microbes (Perrier & Corthésy, 2011). However, the delicate equilibrium at the base of the tolerogenic response can be compromised by environmental triggers, such as viral infections (Bouziat et al., 2017) or intrinsic predisposing factors, leading to inappropriate immune and inflammatory responses. In this context, Celiac disease (CD) is an immune-mediated disorder characterized by an autoimmune enteropathy due to exposure to dietary proteins from wheat, rye and barley, occurring in 1% of individuals worldwide (Leonard et al., 2017). CD belongs to a spectrum

of gluten-related disorders (GRD), which also includes non-celiac gluten sensitivity (NCGS), dermatitis herpetiformis, and gluten ataxia. It also occurs in genetically predisposed individuals bearing the human leukocyte antigen (HLA) DQ2/DQ8. In a subset of genetically susceptible individuals, the ingestion of gluten proteins switches tolerance toward an adaptive immune response with an autoimmune component (Sollid & Jabri, 2013). There is a strong link between CD and autoimmunity given that 5%–10% of patients with diabetes 1 type (T1D) develop CD, whereas, 15%–20% of CD patients have or will develop autoimmune diseases (Cosnes et al., 2008).

2.1 CD DIAGNOSIS

Early diagnosis of the disease in patients with atypical symptoms decreases the risk of serious complications such as intestinal lymphoma. Untreated CD may lead to osteopenia and infertility and may trigger other autoimmune diseases. Therefore, CD should be diagnosed as early as possible. The clinical spectrum of celiac disease is wide, including cases with either typical intestinal (e.g., chronic diarrhea, weight loss) or “atypical” extraintestinal (e.g., anemia, osteoporosis, neurological disturbances) features, and silent forms that are occasionally discovered because of serological screening (Figure 5).

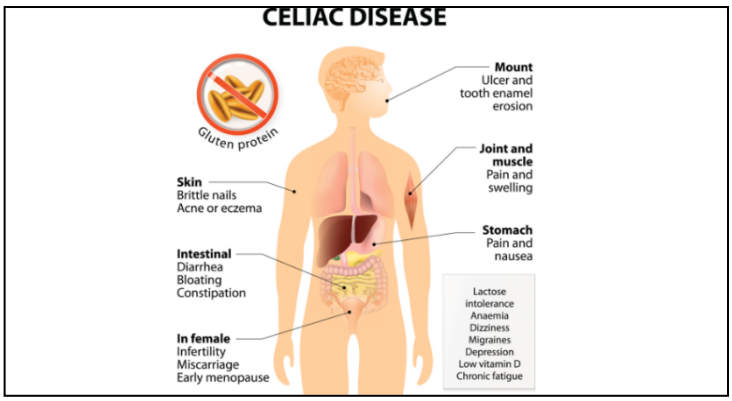

Figure 5. Schematic CD feature. *https://www.gutmicrobiotaforhealth.com/*

The typical jejunal damage associated with active celiac disease is characterized by villous atrophy (Figure 6), crypt hypertrophy, and increased intraepithelial lymphocyte count. The small intestinal biopsy is still considered a key investigation in the current guidelines of North American and European gastroenterological societies.

Indeed, many individuals with celiac disease may have no symptoms and the disease is usually detected by serologic testing of celiac-specific antibodies, which is confirmed by duodenal mucosal biopsies. Both serology and biopsy should be performed on a gluten-containing diet. In the serologic analysis the Immunoglobulin (Ig)A anti-gliadin (AGA), anti-endomysial (EMA) and anti-tissue transglutaminase (TG2) antibodies are considered the best immunologic markers of CD.

The treatment for celiac disease is primarily a gluten-free diet (GFD).

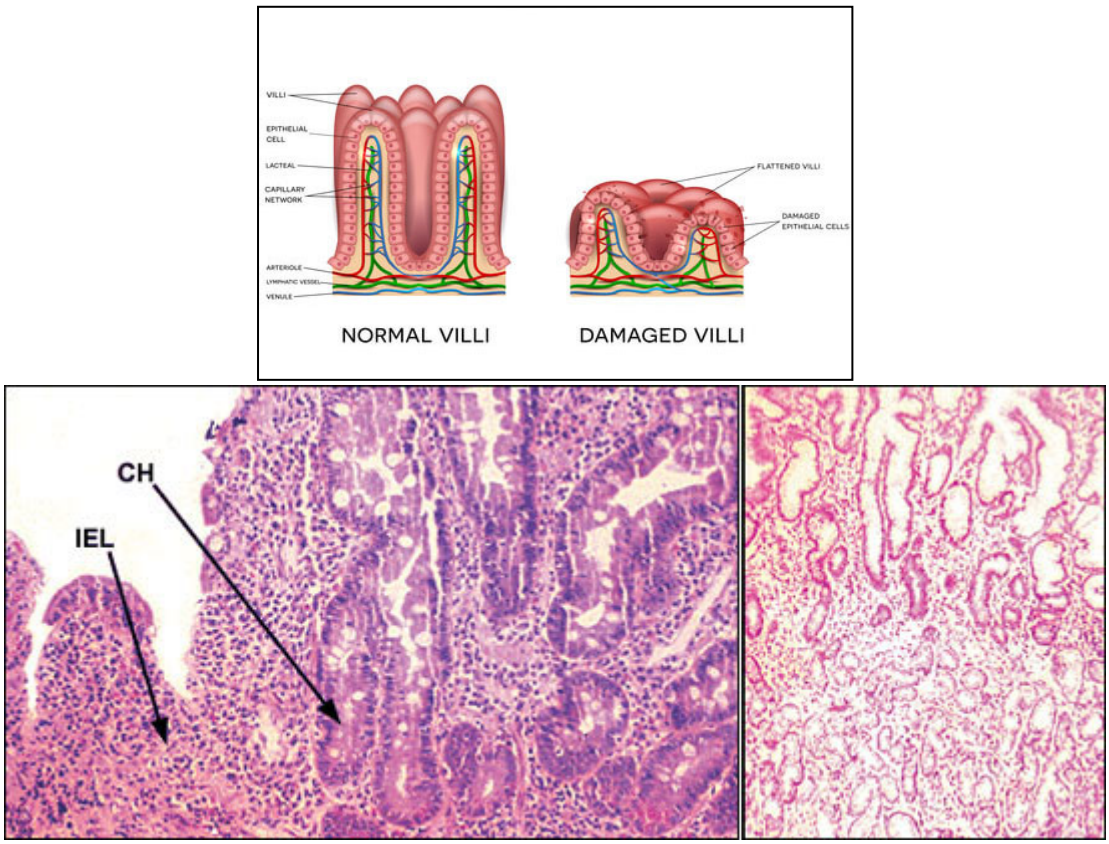

Figure 6. CD features. *Villous atrophy (upper panel; beyongcelic.org); crypt hypertrophy (CH, bottom panel, Nelson DA, 2002).*

2.2 THE PATHOGENESIS OF CD

CD occurs in genetically predisposed people bearing the human leukocyte antigen HLA DQ2/DQ8. However, the presence of HLA-DQ8 is necessary but not sufficient for the CD pathogenesis. Other genetic risk factors, involved in the barrier and immune functions, also contribute to disease susceptibility although accounting for a small portion of the overall risk (Bouziat et al., 2017; Sciurti et al., 2018).

Gluten is made up of gliadins and glutenins, both of which are capable of triggering CD, with gliadins causing an immediate and transient increase in intercellular TJ permeability of intestinal epithelial cells (Drago et al., 2006). Several properties of gluten may explain their toxicity in patients with CD. Indeed, gluten proteins have a high concentration of proline and glutamine residues, which renders them resistant to enzymatic degradation by digestive enzymes, leaving large, potentially immunogenic peptides that may reach the underlying mucosa (Chander et al., 2018). One of these peptides is known as p31-43, or 33-mer, and is highly immunogenic in CD patients. When in the intestinal lumen, gliadin binds the chemokine CXC motif receptor 3 (CXCR3), promoting the activation of MYD88 thus inducing the release of zonulin in the lumen. The latter protein binds the EGFR and PAR2, which complex ignite the signaling pathway leading to zonula occludens protein phosphorylation, responsible for tight junction disassembly (figure 7). When gluten peptides cross the epithelial barrier, the high proline and glutamine content renders them a very good substrates for tissue transglutaminase (TG-2) (Vader et al., 2002), which plays a key role in CD pathogenesis. This is a member of the transglutaminase family characterized by several enzymatic activities ($Ca2^{+}$-dependent transamidating activity, GTPase/ATPase, protein disulfide isomerase, protein kinase) as well as nonenzymatic functions based on its non-covalent scaffold interactions with many cellular proteins (Odii & Coussons, 2014). Due to its multifunctionality, TG2 has been reported to have a complex biology playing a role in a

variety of cellular processes, such as differentiation, survival, apoptosis, autophagy, and cell adhesion (Tatsukawa et al., 2016). Once activated, TG2 deamidates gluten peptides, with this interaction being influenced by the initial imprinting of the innate immune system through the upregulation of IL-15, promoting the $CD4^+$ T cell adaptive immune response (figure 7) (Tang et al., 2009). Thus, TG2 converts glutamines of the gluten peptides into negatively charged glutamic acid residues. These negatively charged residues, together with the presence of proline residues, increase the ability of peptides to bind the HLA-DQ2/8 on APCs (Bergseng et al., 2015; Molberg et al., 1998), which are than presented to gluten specific $CD4^+$ T cells, inducing their prompt activation, migration to the small intestinal lamina propria, production/release of proinflammatory cytokines, such as IFNγ, metalloproteases, and keratinocyte growth factor by stromal cells, which induces cryptal hyperplasia and villous blunting, secondary to intestinal epithelial cells death induced by intraepithelial lymphocytes (IELs) (figure 7) (Pagliarini et al., 2015). Additionally, active CD is also characterized by membrane bound IL-15 overexpression on enterocytes, causing overexpression of the natural killer (NK) receptors CD94 and NKG2D by $CD3^+$ IELs (Jabri & Abadie, 2015). Crypt hyperplasia has been hypothesized to be the consequence of an imbalance between continuous tissue damage, due to the mucosal autoimmune insult described above, and inability of the stem cells to compensate. The epithelial damage is mediated by cytotoxic intraepithelial lymphocytes which recognize ligands produced by inflammatory stimuli on the surface of the intestinal epithelium (Green & Jabri, 2003). Indeed, gliadin peptide 33-mer (p31–43) is responsible for the activation of innate immunity of the intestinal epithelial cells (Maiuri et al., 2003). Thus, the gliadin peptides can also directly stimulate the immune response of macrophages and dendritic cells through pattern recognition receptors (PRR), such as toll-like receptors (TLRs) 4 (Visser et al., 2009), which leads to the maturation of these cells and the secretion of pro-inflammatory cytokines (e.g., IL-1b, IL-8, TNFa and MCP-1). Consequently, the adaptive immune response directed

against gluten is enhanced, and the intestinal permeability is increased (Schuppar et al 2009).

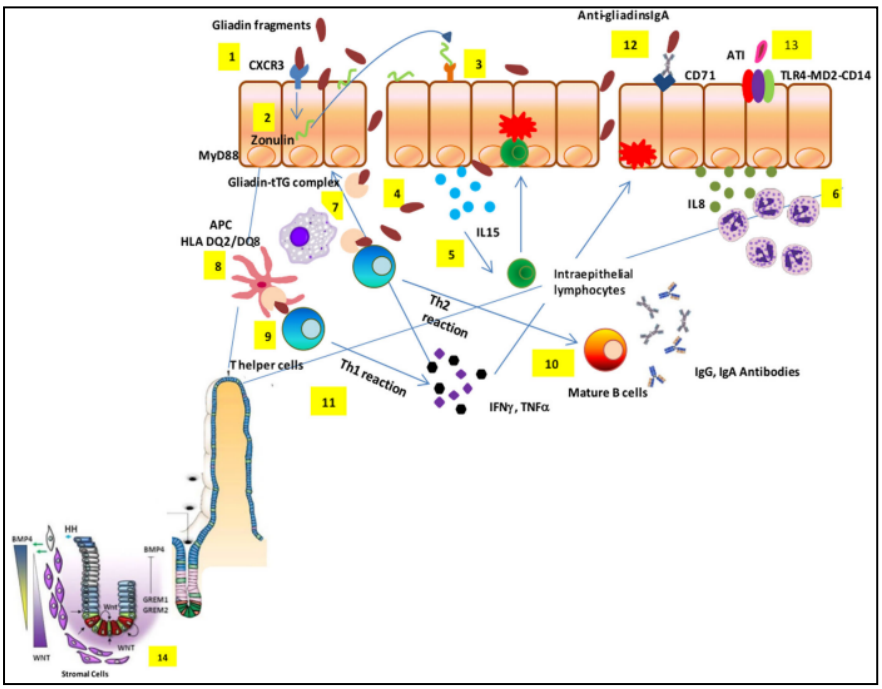

Figure 7. CD pathogenesis. *gliadin peptide (p31-43 or 33mer) interacts with CXCR3 on the apical side of epithelium (1) inducing a myeloid differentiation primary response 88-dependent release of zonulin (2). Zonulin interacts with the intestinal epithelium and triggers increased intestinal permeability (3). Functional loss of the gut barrier facilitates gliadin peptide translocation from lumen to the lamina propria (4). Gliadin peptides allow the release of IL-15, keratinocyte growth factor, and IL-8 (5), with consequent recruitment of neutrophils in the lamina propria (6). Contemporaneity, alpha-amylase/trypsin inhibitors engage the Toll like receptor 4–MD2–CD14 complex with subsequent upregulation of maturation markers and release of proinflammatory cytokines (7). Then there are the innate immune-mediated apoptosis of intestinal cells with subsequent release of intracellular tissue transglutaminase, gliadin peptides are partially deamidated (8). Deamidated gliadin is recognized by DQ2/8+ antigen presenting cells (9) and then presented to T helper cells (10). T helper cells guide the activation and maturation of B cells, producing IgM, IgG, and IgA antibodies against tissue transglutaminase (11). T helper cells also produce IFNγ and TNFα (12), which in turn further increase gut permeability and, together with T killer cells, initiate the enteropathy. Damaged enterocytes express CD71 transporter also on their apical side, resulting in retro-transcytosis of secretory IgA-gliadin complexes (13), thus potentiating gluten trafficking from gut lumen to lamina propria. At the end the interaction between CD4+ T cells in the lamina*

propria with gliadin induces their activation and proliferation, with production of proinflammatory cytokines, metalloproteases, and keratinocyte growth factor by stromal cells, which induces crypt hyperplasia and villous blunting secondary to intestinal epithelial cell death induced by intraepithelial lymphocytes. The hyperplastic crypts (14) are characterized by an expansion of the immature progenitor cells compartment (WNT) and downregulation of the Hedgehog signaling cascade. An increased number of stromal cells known to be part of the intestinal stem cell niche and increased levels of bone morphogenetic protein antagonists, like Gremlin-1 and Gremlin-2, may further contribute to the crypt hyperplasia present in celiac disease.

3. THE GUT MICROBIOTA

In the last decade, epidemiological, physiological and omics-based studies, in addition to cellular and *in vivo* studies, indicated that a considerable part of the environmental influence on human health and disease risk may be mediated or modified by microbial communities in our body (Marchesi et al., 2016). These communities, named microbiota, include a large number of interacting bacteria, bacteriophages, archaea, eukaryotic virus and fungi that live together on human surfaces and in all body cavities. Most of them are commensal or mutualistic microorganisms. The whole intestinal microbial genes (the microbiome) in an individual represents a genetic repertoire that is more than one order of magnitude higher in genes than the human genome (Marchesi et al., 2016).

Recent evidence indicates that intestinal microbiota plays a crucial role in maintaining the homeostasis of the human body. This studies suggest that microbiota may contribute to the pathogenesis of several metabolic disease such as obesity, type 2 diabetes, non-alcoholic liver disease, cardio-metabolic diseases and malnutrition (Marchesi et al., 2016). It is important to note that most microorganisms inhabiting humans reside in the intestine, and the gut microbiota composition is influenced by the mode of birth, lifestyle, infant feeding, and medication of the human host. The gut microbiota plays an important role in the training of immunity, digesting food, modifying drug action and metabolism, regulating gut endocrine

function and neurological signalling, eliminating toxins, and producing numerous compounds that influence the host (Lynch & Pedersen, 2016). For example, there are indications for a role of the gut microbiota in mediating some of the environmental effects in obesity pathogenesis. This evidence indicated that there is a transferable obesity-associated microbiota that can induce weight gain in lean mice (Turnbaugh et al., 2006). Subsequent studies have also shown differences in the gut microbiota of individuals with obesity and lean individuals (Turnbaugh et al., 2006).

The microbiota integrity and its dynamic interactions with the body are essential for maintaining a healthy state and homeostasis while its alterations may contribute to the development of diseases. Indeed, it has been hypothesized that alterations in the gut microbiota play a pathogenic role in celiac disease (Collado et al., 2007), and recent studies suggest that a dysbiosis of the gut microbiota leads to the activation of innate immunity in patients with CD (Szebeni et al., 2008).

Bacterial products on the gut surface are detected by specific receptors for the pathogen-associated molecular pattern (PAMP) called TLRs. Each of these receptors is able to recognize a specific bacterial product which can lead to the activation of various signaling pathway in the cell (Round & Mazmanian, 2009). The innate immune reaction is immediate and is usually directed toward microbial antigens which bind the toll-like receptors. In CD patients this reaction is directed toward gluten (Garrett et al., 2010; Meresse et al., 2012). A recent study demonstrated an increased expression of TLR4 and TLR2 in both inflammatory bowel diseases (IBD) and CD, suggesting a role of dysbiosis in the initiation of intestinal barrier damage (Szebeni et al., 2008).

The relationship between the gut and its microbial content allows the identification of dangerous and harmless bacteria as well as food antigens (Nagler-Anderson, 2001). In addition, the microbiota contributes to maintaining both the intestinal barrier function, by increasing the proliferation of epithelial cells, and the integrity of the intestinal epithelium

through the translocation of proteins forming TJs and stimulating the expression of genes involved in the maintenance of desmosomes (Kim et al., 2010). As a result, it also regulates the development of the vascular architecture of the villi (Stappenbeck et al., 2002).

3.1 CD TREATMENT

Actually, the only treatment for celiac disease is life-long gluten-free diet (GDF).

Early diagnosis of celiac disease is very important to prevent some complication that could be irreversible, such as growth retardation, osteoporosis and abnormal dentition (Freeman et al., 2011)). Many studies suggest that delaying and gradually introducing gluten in the diet can reduce the risk of celiac disease development in the childhood (Parzanese et al., 2017)

3.2 CD AND PROBIOTICS

GFD represents a very hard problem for many patients because traces of gluten are found in the most of processed foods, and a strict gluten-free diet inevitably limits the social activities of patients. Furthermore, GFD may be rich in high glycemic index foods which can increase insulin resistance and the risk of obesity and cardiovascular diseases. In the last decade, many new therapies have been suggested to improve GFD or to replace it. The microbiota and the related dysbiosis in CD patients have shifted the focus of new therapies for CD regarding the use of probiotics. Indeed, recent studies suggest that probiotics and prebiotics administration could be useful in the treatment of celiac disease patients (de Sousa Moraes et al., 2014). Probiotics administration could modulate the composition and functions of the intestinal microbiota, both to defer or to avoid the onset of CD, and could also be useful following a GFD when the normal microbial composition has not been completely restored. Benefits could come from the regulation of the immune response, the degradation of toxin receptors, the competition for nutrients, and the production of inhibitory substances against pathogens. (Marasco et al., 2020).

4. CYSTIC FIBROSIS AND CD

Several lines of evidence led to the hypothesis of a link existing between the two human disorders such as CD and Cystic fibrosis.

4.1 CYSTIC FIBROSIS

Cystic fibrosis (CF) is a human disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CF is the most common rare disease in the Caucasian population (Cutting, 2015), mainly affecting the lung, but also gut, kidneys, pancreas, and liver (Quittner & Li-Rosi, 2020). Mutated CFTR causes salt water balance dysfunction, resulting in dehydration of the secretions (thick mucus) and excessive loss of salt in sweat. Unfortunately, there is not yet a cure for CF (Massie & Delatycki, 2013). A defective CFTR causes an increase of reactive oxygen species (ROS), early activation of TG2, epithelial stress, autophagy inhibition (Ferrari et al., 2017; Luciani et al., 2010) and, TG2-mediated increased nuclear translocation of NF-κB, resulting in enhanced levels of pro-inflammatory cytokines (Luciani 2010).

CFTR is a membrane protein belonging to the ABC transporter family functioning as a chloride/anion channel in epithelial cells. It is composed by two nucleotide-binding domains (NBDs) in tandem with two transmembrane domains (TMDs; figure 8) (Meng et al., 2017).

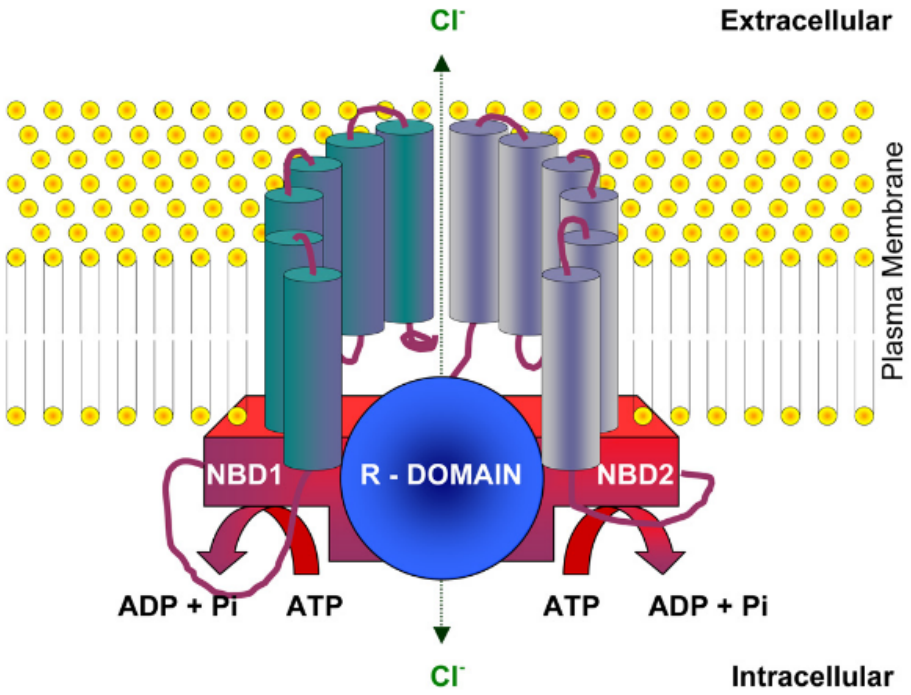

Figure 8. Structure of CFTR protein. *CFTR is composed of two transmembrane domains, two nucleotide binding domains (NBD1 and NBD2) and a regulatory domain (R). CFTR chloride channel can be activated through phosphorylation of the R domain and by ATP binding to and hydrolysis by NBDs.*

There are over 1500 different mutations associated to cystic fibrosis (http://www.genet.sickkids.on.ca). Most mutations identified in CF patients occur in the first nucleotide binding domain (NBD1), while very few occur in the NBD2, of CTFR protein. The deletion of phenylalanine at position 508 (F508del) is the most common mutation in CF (Quittner & Li-Rosi, 2020). Homozygous F508del (CFTR-ΔF508) is related to defective intracellular processing and trafficking (Ward & Kopito, 1994) and results in a loss of function mutation. Importantly, the mutated protein is retained in the endoplasmic reticulum (ER) and rapidly degraded. As a result, CFTR-ΔF508 fails to reach the apical membrane(Cheng et al., 1990).

4.2 CF AND CD CO-MORBIDITY

The co-morbidity CF and CD were reported since 1960s in a large cohort of patient (Fluge et al., 2009). There are 4% prevalence of positive serological marker of CD (Fig.9). The NF-κB activation in CF leads to increased levels of pro-inflammatory cytokines, such as IL-17A, IL-21 and IL-15, involved in the intestinal homeostasis. These concerns led to the hypothesis that CFTR is potentially involved in the pathogenesis of CD.

Patients	N°	Percentage
Cystic Fibrosis	288	
CF patients with:		
Specific antibody response to gluten	12	4.16%
Diagnosis of CD **(a)**	8	2.78%

a. Data from children with Cystic Fibrosis who have received diagnosis of Celiac Disease

Patient	CF Genotype	CI	tTG IgA	EMA IgA	Duodenal histology^	HLA-DQ
P1	F508del/F508del	104	76.1	+	T3b	DQ8
P2	2789+5G>A/Y849X	106	21.4	+	T3c	N.A.
P3	G542X/2184insA	94	17.9	+	T3a	DQ2
P4	F505del/D1152H	44	>200	+	T3c	DQA1*03
P5	F508del/L732X1	80	>200	+	T3c	DQ2
P6	171711G>A/D579G	67	23.3	+	T3b	DQ2
P7	D1152H/D579G	15	>200	+	T3c	DQ2
P8	F508del/F508del	128	161.6	+	T3a	DQ2

b. Data from children with Cystic Fibrosis who have shown fluctuant serological anti-tissue anti-transglutaminase and anti-endomysial antibodies above the cut-off level at least once.

Patient	CF Genotype	CI	tTG IgA*	EMA IgA*	Duodenal histology^	HLD-DQ2
P9	N1303K/G1244E	98	+	+	N.A	DQ2
P10	F508del/G542X	109	+	+	T0	DQ2
P11	F508del/F508del	85	+	+	N.A	DQA1*05
P12	G542X/4016insT	79	+	+	T0	DQB1*06

N.A. Not available; CF Cystic Fibrosis; tTG anti-tissue transglutaminase; EMA anti-endomysial antibodies ^, Marsh classification of intestinal lesion; *, highest antibody titer above the cut-off level registered at leat once during the screening

Figure 9. *Prevalence of CD in Paediatric group of 288 patients with CF at the Paediatric Cystic Fibrosis Care Unit of the University of Naples Federico II during the last 10 years.*

AIMS

5. AIMS

The aims of my PhD project were:

- ✓ to characterize *in vivo* pre-clinical mice models of celiac disease. In vivo models can represent an advantageous tool to study CD pathogenesis and to define new therapeutic strategies

- ✓ to decipher the molecular mechanisms underlying the onset and development of this disorder. Particularly, to unveil the hypothetical link between CF and CD. To this aim, I focused on CFTR protein function *in vivo* under gliadin stimulation, to induce CD

- ✓ finally, due to the key role played by the microbiota and its dysbiosis in several inflammatory diseases such as IBD and CD, I evaluated the impact of probiotics administration in an *in vivo* preclinical CD mouse model.

MATHERIAL AND METODS

MATERIALS AND METHOD

6.1 Mice and treatments

- BALB/c mice (background BALB/cAnNCrl; Papista et al, 2012) were purchased from Charles River (Calco(LC), Italy). Three-generation gluten-free diet (Mucedola srl, Milan), male and female, were challenged with gliadin for 4 weeks (Papista et al., 2012; Villella et al., 2019). Mice were challenged via gavage for 4 weeks with (i) vehicle alone or (ii) gliadin (SigmaAldrich); 5 mg/daily for 1 week and then 5 mg/daily thrice a week for 3 weeks; (Galipeau et al, 2011; Papista et al, 2012; Larsen et al, 2015; Moon et al, 2016). At the end of the fourth week, mice were challenged every day via gavage for two weeks with gliadin in presence or absence of Probiotic 1 ($5,7x10^5 \pm 2.0X10^5$/day) or Probiotic 2 (P2: $8,5x10^5 \pm 2.0X10^5$/day /day) formulations. (n = 6 mice per group of treatment). All described mice, male and female were 8 weeks old.

- Prediabetic NOD (Non-obese diabetic) mice were purchased from Charles River (Calco (LC), Italy) . Diabetes incidence was followed weekly measuring blood glucose levels with a Contour glucose meter (Bayer; US). At time of 12–13 weeks, female mice with manifested diabetes (> 250 mg/dl) were challenged as described in (i) or (ii).

- NOD.scid AB0nullDQ8 mice (NOD DQ8tg, transgenic mice expressing HLA-DQ8 in an endogenous MHC class II-deficient background were backcrossed to NOD mice for 10 generations and intercrossed to produce congenic NOD AB0 DQ8 mice (Galipeau et al., 2011). NOD-DQ8 mice, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were weaned and maintained on a low-fat (4.4%), gluten-free diet (Mucedola srl, Milan), and bred in a conventional, specific

pathogen-free colony at the San Raffaele Scientific Institute SOPF animal house (Milan, Italy). Mice were challenged as described in (i) or (ii).

- CF mice homozygous for the F508del-CFTR in the FVB/129 outbred background (Cftrtm1EUR, F508del, FVB/129, abbreviated CF) and Wild Type littermates were obtained from Bob Scholte, Erasmus Medical Center Rotterdam, The Netherlands. Transgenic KO Cftr mice (B6.129P2-KOCftrtm1UNC, abbreviated CftrKO), and Wild Type littermates, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were treated as previously described in (i) or (ii) (Villella et al., 2019).

- Balb/c mice were obtained from Charles River (Calco). 8 weeks old of three-generation gluten free mice (Mucedola), for a total of 36 mice, were randomly divided into 6 groups (G1÷G6), composed of 6 mice/group. The G1 was challenged with a gluten free diet for all along with the time of the experiment; the G2÷G4 were challenged via gavage with gliadin (Sigma; 5mg/daily for 1 week, then 5 mg/daily thrice a week for 3 weeks) for 4 weeks (Villella et al, 2019; Papista et al, 2012). At the end of the fourth week, mice from G2÷6 were challenged with gliadin alone (G2) or in combination with Probiotic 1 (P1; G3) or Probiotic 2 (P2; G4), or with P1 (G5) or P2 (G6) alone, every day via gavage, for another two weeks (P1 2mld/day; P2 3mld/day). A schematic representation of treatments is reported in Figure 10.

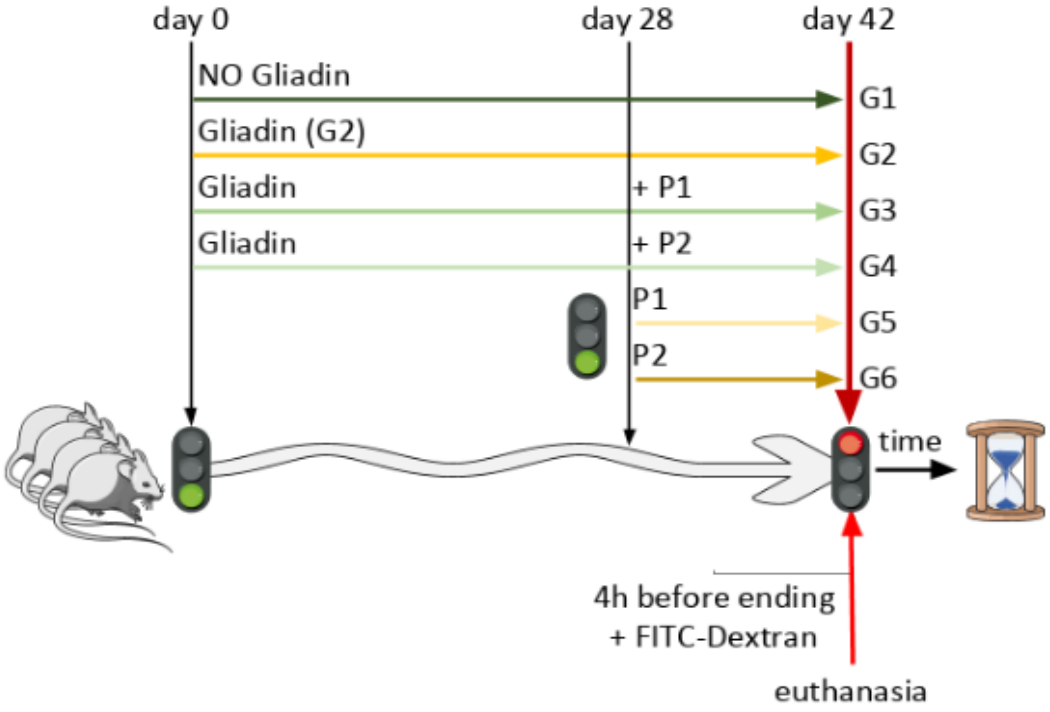

Figure 10. *Schematic representation of the experimental procedure*

At the end of every last daily treatment, mice were anesthetized with avertin (tribromoethanol, 250 mg/kg, Sigma-Aldrich, T48402) and euthanized; the intestines were collected.

These studies and procedures were approved by the local Ethics Committee for Animal Welfare (IACUC No 583, 849) and conformed to the European Community regulations for animal use in research (2010/63 UE).

6.2 Ussing chamber

Chambers for mounting either transwell cell cultures or mouse tissue biopsies were obtained from Physiologic Instruments (model P2300, San Diego, CA, USA). Chamber solution was buffered by bubbling with identical Ringer solution on both sides and were maintained at 37°C, vigorously stirred, and gassed with 95% O_2/5% CO_2. Cells or tissues were short circuited using Ag/AgCl agar electrodes. A basolateral-to-apical chloride gradient was established by replacing NaCl with Na-gluconate in the apical (luminal) compartment to

create a driving force for CFTR-dependent Cl secretion (figure 11). To measure stimulated Isc, the changed sodium gluconate solution, after stabilization, was supplied with 100 mM amiloride. Agonists (forskolin) were added to the bathing solutions as indicated (for a minimum of 5 min of observation under each condition) to activate CFTR channels present at the apical surface of the epithelium (either cell surface or lumen side of the tissue), and CFTRInh-172 (10 mM) was added to the mucosal bathing solution to block CFTR-dependent Isc. Short-circuit current [expressed as Isc (IA/cm2)] and resistance were acquired or calculated using the VCC-600 transepithelial clamp from Physiologic Instruments and the Acquire&Analyze23 software for data acquisition (Physiologic Instruments), as previously described (Gondzik & Awayda, 2011; Marchelletta et al, 2013; Tosco et al, 2016; Romani et al, 2017;Villella et al., 2019).

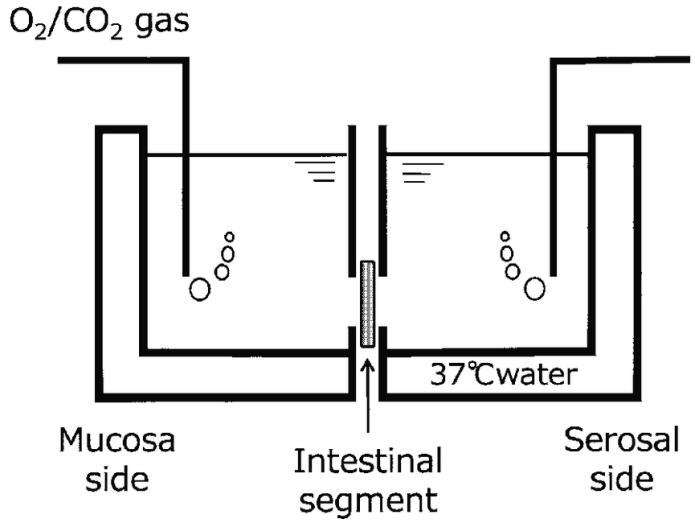

Figure 11: schematic representation of Ussing Chamber

6.3 Permeability assay

The FITC-D4000 test in treated BALB/c mice (as described in mice and treatments section) was performed as previously described (Volynets et al., 2016). Briefly, FITC-D4000 (Sigma-Aldrich) was administered to mice by oral gavage at a concentration of 600 mg/kg body weight and a volume of 200–300 ml, using a stock solution of 50 mg/ml. After gavage, the

mice remained in the metabolic cages until the experiments were completed and the mice were euthanized. One hour after gavage, the animals were anesthetized and blood was taken by cardiocentesis, heparinized, and then centrifuged (10 min, 12,000 g,4 °C). Plasma was light protected and stored at -80°C for photometric analysis of FITC-D4000. Each plasma was diluted in an equal volume of phosphate buffered saline (PBS, pH 7.4). Standards (range 50–0.312 µg/ml) were obtained by diluting the FITC-D4000 gavage stock solution in PBS. An amount of 100 µl of both diluted animal samples and standards, as well as blanks (PBS and diluted plasma from untreated animals), were transferred into black 96-well microplates. Analysis of the FITC-D4000 concentration was carried out with a fluorescence spectrophotometer (Multi-Detection Microplate Reader) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The same analysis was previously described in (Villella et al., 2019).

6.4 Real-Time and reverse transcription analysis

Trizol reagent (Invitrogen) was used to extract total RNA. The AMV Reverse Transcriptase kit (Promega; Madison, WI, US) was used to produce cDNA following the manufacturer's recommendations. Quantitative PCR reactions were performed by using C1000Touch CFX96 Biorad thermocycler. The Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used to produce fluorescently labeled PCR products during repetitive cycling of the amplification reaction and the melting curve protocol was used to check for probe specificity. Primer sets for all amplicons were designed using the IDT PrimerQuest Tool (https://eu.idtdna.com/Primerquest/Home/Index).The sequences of mouse primers were as follow:

primer	Sequenze (forward/reverse)
IL-15	CAGCAAGGACCATGAAGA/GGCTGAGTTCCACATCTAAC

ATF4	GTTTAGAGCTAGGCAGTGAAG/CCTTTACACATGGAGGGATTAG
ATF6	GATGGTGACAACCAGAAAGA/TGGAGGTGGAGGCATATAA
CFTR	GTAGACACACCAGGAGTCTG/AGGAGGACAGGGATGATAAG
CLD15	GGGACCCTCCACATACTT/CATACTTGGTTCCAGCATACA
CLD2	CCTCGCTGGCTTGTATTATC/AAAGACTCCACCCACTACA
GAPDH	TTCAACGGCACAGTCAAG/CCAGTAGACTCCACGACATA
IL-17a	CGCAATGAAGACCCTGATAG/CTTGCTGGATGAGAACAGAA
INF_{γ}	CCACATCTATGCCACTTGAG/CTCTTCCTCATGGCTGTTTC
OCLN	TCTTTGGAGGAAGCCTAAAC/CTGCTCTTGGGTCTGTATATC
TG2	AAGAGCGAAGGGACATACT/TGAGCACAGACCCATCTT
XBP1	AGTCCGCAGCAGGTG/GGTCCAACTTGTCCAGAATG

The result of the fluorescent PCR was expressed as the threshold cycle (CT). The ΔCT is the difference between the CT for the specific mRNA and the CT for the reference mRNA, GAPDH. To determine relative mRNA levels, 2 was raised to the power of ΔΔCT (the difference between the ΔCT from treated cells and the CT from untreated cells).

6.5 Immunoblot

The whole small intestine lysates were obtained by using the Cell Lytic buffer (Sigma) supplemented with a protease inhibitors cocktail (Sigma) plus phosphatases inhibitors (Na3VO4 1 mM; NaF 10 mM), and resolved by electrophoresis through SDS-PAGE, and electroblotted onto nitrocellulose (Protran, Sigma) membranes. Membranes were incubated with indicated primary antibodies in 5% non-fat dry milk (Bio-Rad) in PBS plus 0.1% Tween20 overnight at 4°C. Primary antibodies were: anti-CFTR (clone M3A7 Abcam ab4067) 1:500, anti-TG2 (NeoMarkers) 1:750, and anti-□Actin (Cell Signaling) 1:2000.

Detection was achieved using horseradish peroxidase-conjugate secondary antibody (1:5000; Jackson ImmunoResearch; Cambridge, UK) and visualized with ECL plus (Amersham Biosciences; Amersham). Images were acquired by using a ChemiDoc™ Touch Imaging System (Bio-Rad) and analysed by Image Lab software (Bio-Rad), as previously described.

6.6 ELISA

ELISA was performed on tissue samples using standard ELISA kits (R&D Systems) for IL-15, IL-17A, IFN-*γ*. According to the manufacturer's instructions, samples were read in triplicate at 450 nm in a microplate Reader (Bio-Rad, Milan, Italy) using Microplate Manager 5.2.1 software. Values were normalized to protein concentration evaluated by Bradford analysis.

6.7 H/E staining

After surgical removal of the small intestine, samples were fixed in formalin at room temperature, dehydrated and embedded in paraffin. 8□m thick sections were collected by using a microtome (Leica). All sections were mounted on slides, stained with haematoxylin and eosin (Bio Optica) and images were acquired by using a Nikon Eclipse Ci Microscope, a Plan APO 10X Objective, and the NIS-Elements Software (Nikon).

6.8 Probiotics formulations

Probiotics were supplied by PROBIOTICAL SpA, in lyophilized powder. The P1 formulation contains two strains of Bifidobacterium breve, the B632 (DSM 24706) and BR03 (DSM16604) at 2x109 live cells (AFU)/g, while the P2 formulation contains the Lactobacillus plantarum LP14 (DSM 33401), L. casei subsp. paracasei LPC09 (DSM 24243) and the Lactobacillus rhamnosus LR04 (DSM 16605) at 3x109 live cells(AFU)/g. The study materials

were analyzed by Probiotical Research srl, Novara, Italy, via flow cytometry (ISO 19344:2015 IDF 232:2015) to confirm target cell count. P1 or P2 were resuspended in PBS and administrated as described.

6.9 Statistical analysis

All experiments were performed at least in triplicate and statistical analysis was performed using GraphPad Prism 6. The Student's t test and ANOVA was used to determine statistical significance. A p-value of equal to or less than 0,05 was considered significant.

RESULTS

RESULTS

7.1. IN VIVO MODELS OF GLUTEN SENSITIVITY

The identification and development of *in vivo* pre-clinical models of celiac disease can represent a useful tool to study disease pathogenesis and to define new therapeutic strategies. Such in vivo models could allow to decipher the molecular mechanisms through which prior activation of the innate response can pave the way for T cell response upon gliadin exposure, and potentially unravel the contribution of the intestinal microbiota to the pathogenic cascade leading to celiac disease.

To this aim, I screened the literature to identify appropriate animal models sensitive to gluten.

Therefore, I selected 3 mouse models of CD:

- Balb/c mice inbred for at least three generation and fed with a gluten-free diet. Gluten sensitivity was induced by feeding mice with food containing gluten for 30 days (Papista et al., 2012), ***model 1***;
- NOD mice fed either with a gluten-free or standard diet (Larsen et al 2015), ***model 2***;
- Gluten sensitive human leukocyte antigen (HLA) DQ8 transgenic mice (Galipeau et al., 2011), ***model 3***.

Then, I tested the response of these mice models to gluten exposure according to literature (Moon et all 2016; Papista et al 2012; Galipeau et al., 2011). To this aim, gluten sensitivity was induced in Balb/c mice by feeding them with a gluten free diet (commercial food pellets) for three generation (model 1). Mice were then challenged with gliadin for 4 weeks, while mice fed with a gluten-free diet were used as matched controls.

NOD mice (model 2) naturally develop diabetes (females) at about 12 weeks of age. Therefore, these mice were challenged with or without gliadin for four weeks, upon diabetes occurrence.

NOD DQ8 mice (model 3) maintained on a low-fat (4.4%) gluten-free diet, were also challenge with or without gliadin for 4 weeks.

7.2 Gliadin increases intestinal permeability, TG2 levels, and cause villous atrophy *in vivo*

Under physiological conditions, access of gliadin to gut associated lymphoid tissue is prevented by competent intercellular tight junctions (TJs), thus limiting the passage of macromolecules through the intestinal epithelial barrier. However, in predisposed individuals, gliadin exposure leads to TJ disassembly, thus increasing intestinal permeability and chronic inflammation. To test this hypothesis, I evaluated the permeability of intestinal barrier in gluten-free mice exposed to gliadin (model 1), as described in the previous section, by using FITC-conjugated Dextran. To this aim, gluten sensitive mice (GSM) fed for at least three generation with a gluten-free diet) were gavaged with gliadin for 4 weeks. At the last day of treatment, mice were orally gavaged with a single dose of FITC-Dextran for 4h (44mg/100g body weight), mice were sacrificed and blood was than collected. Fluorescence of isolated plasma was measured and used to evaluate the permeability of the intestinal barrier. Data reported in figure 12A clearly show an increased intestinal permeability of mice challenged with gliadin, compared to matched controls.

TG2 is also a very important actor in CD, deamidating gliadin peptides, thus increasing their binding affinity to the disease-predisposing human leukocyte antigen (HLA) DQ2 and DQ8 molecules, and enabling a strong immune response to be launched. To confirm the involvement of TG2, a western blotting analysis was performed on lysates from the small intestine of GSM exposed to gliadin. Data reported in figure 12B show an enhanced expression of TG2 in mice challenged with gliadin compared to controls.

The CD-associated immune response leading to chronic intestinal inflammation determines shortening of the villi lining the small intestine, resulting in villous atrophy. This condition was also evident in the mouse model 1 exposed to gliadin, as shown in figure 13.

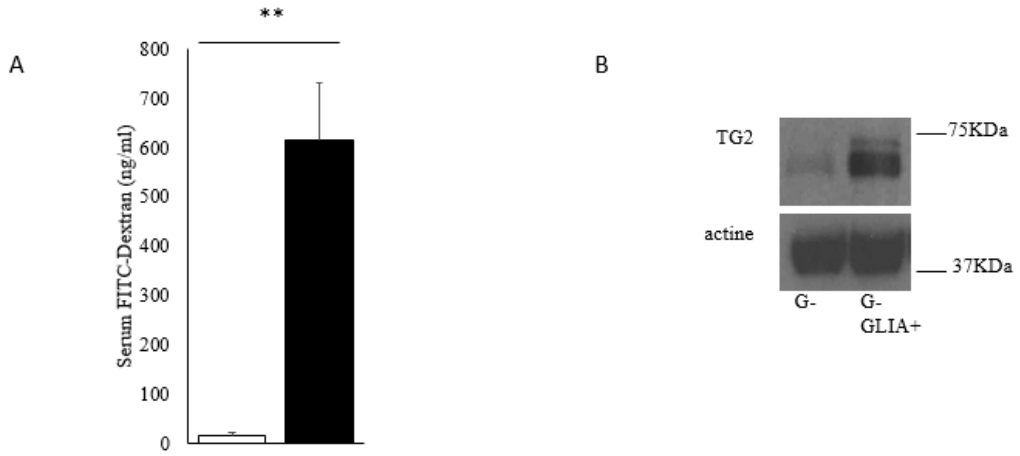

Figure 12. Intestinal permeability and TG2 expression in Balb/c mice challenged with gliadin. *GSM were challanged with gliadin and intestinal permeability was evaluated by monitoring the release of FITC-Dextran in the plasma (A). Plasma concentration of FITC-Dextran was measured 4h after gavage. n=3 mice per group of treatment. T-test** $p<0.05$. B) The expression levels of TG2 was evaluated in intesinal cell lysates from gliadin exposed and matched control mice, by western blotting analysis. Images are representative of experiments performed in triplicete. Actin was used as loading control.*

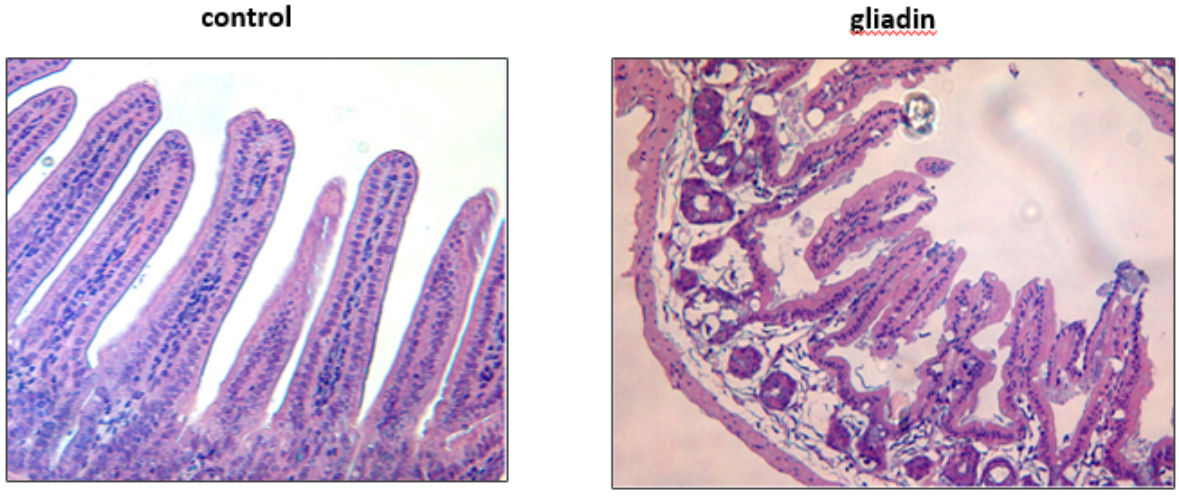

Figure 13. H/E staining of small intestine*. GSM were exposed or unexposed to gliadin for 4 weeks, and H/E staining was performed on small intestine sections.*

The same treatment were done in mice gliadin non-sensitive. In this mice there is no evidence of CD development (data not show).

7.3 Activation of adaptative and innate response *in vivo*

CD is a T-cell mediated disease, in which gliadin derived peptides activate lamina propria infiltrating T lymphocytes which release proinflammatory cytokines. IL-15, exerts many biological functions essential for the maintenance and function of multiple cell types. The dysregulated expression of IL-15 contributes to breaking oral tolerance to gluten and lead to CD pathology. The expression/release of this pro-inflammatory cytokine was than evaluated in small intestine lysates from GSM exposed to gliadin, model 1, compared to matched controls challenged. Figure 14A (left panel) shows a clear increase of IL-15 gene expression compared to control, measured as mRNA levels. Moreover, the production/release of the cytokine is also confirmed by the enhanced protein level evaluated by ELISA, in small intestine lysates of gliadin challenged mice compared to controls (Fig.14B, left panel).

In parallel, the expression of two other proinflammatory cytokines, IL-17A and IFNγ, was also evaluated in the same experimental conditions.

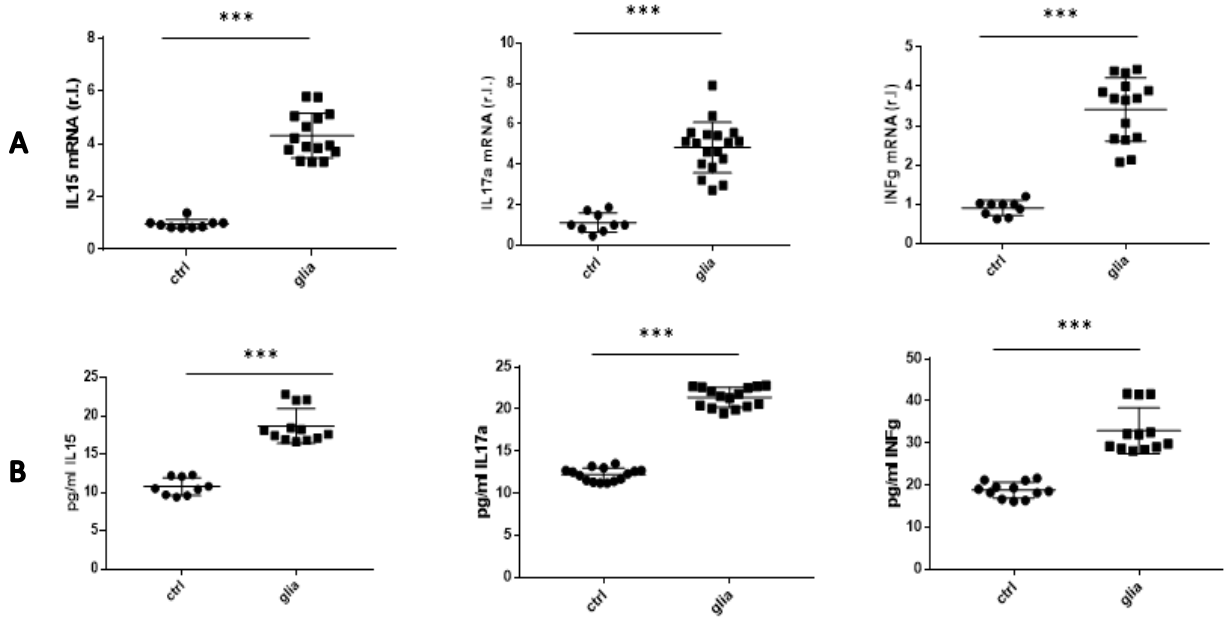

Figure 14. The gluten sensitive Balb/c mouse model. *GSM were challenged with or without gliadin for 4 weeks, and the expression of IL-15, IL-17A or INF$_\gamma$ was evaluated at both mRNA (A) and protein (B) level, in homogenate small intestines, by qRT-PCR and ELISA, respectively. T-test ***P<0.001.*

IL-17A mainly promotes the generation of other pro-inflammatory cytokines and chemokines, which leads to the attraction of neutrophils and macrophages to the inflammation site, while IFNγ represents the primary effector of epithelial permeability in CD. As reported in figure 13, both cytokines were efficiently upregulated at both mRNA (Fig.14A, middle and right panels, respectively) and protein (Fig.14B, middle and right panels, respectively) levels. Collectively these data indicate a proinflammatory condition in small intestine of GSM exposed to gliadin, compared to matched controls.

Then I evaluated the production of the same pro-inflammatory cytokines also in the second model of gluten sensitivity mice, the non-obese diabetic (NOD) female mice (model 2). To this aim, the spontaneous development of autoimmune type-1 diabetes was evaluated by measuring the blood glucose levels (weekly) within 12 weeks. Next, upon diabetes induction, mice were orally administered with gliadin, and the levels of IL-15, IL17A or IFNγ were

evaluated at both mRNA and protein levels, by qRT-PCR and ELISA, respectively, and compared to matched controls.

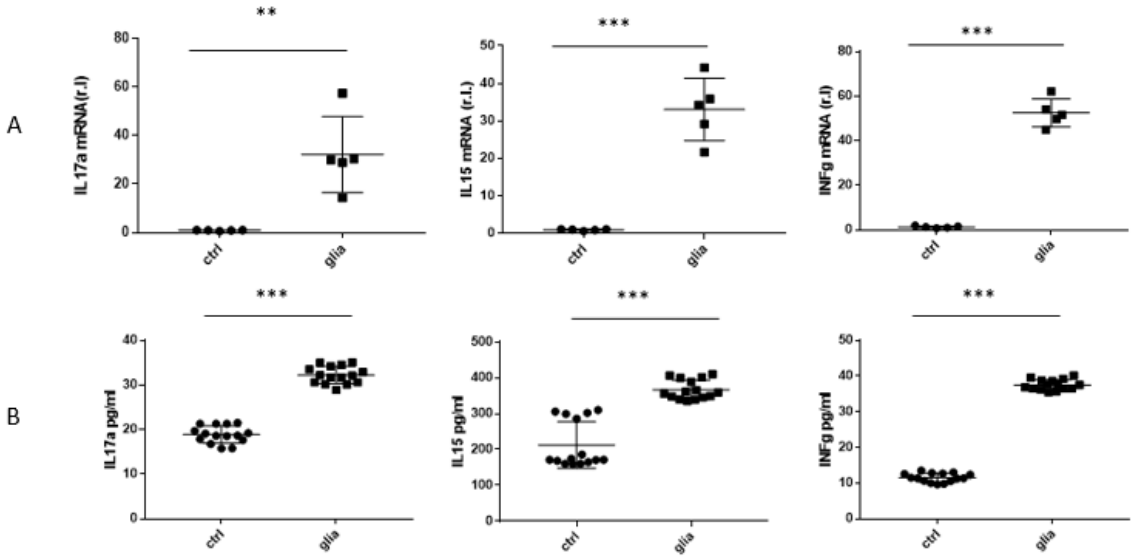

Figure 15. The NOD mice model. *Upon diabetes induction, animals were challenged with or without gliadin for 4 weeks, and the production of IL-15, IL-17A and INF$_\gamma$ was evaluated at both mRNA (A) and protein (B) levels, by qRT-PCR or ELISA, respectively, in homogenate small intestines. t test ***P<0.001, **P<0.05.*

Data shown in Figure 15 clearly confirm an enhanced production of the pro-inflammatory cytokines stimulated by gliadin exposure, compared to controls, also in this *in vivo* model. Finally, the same analysis was also performed by using the selected *in vivo* model 3. To this aim, NOD mice expressing the CD-predisposing HLA molecule DQ8 (NOD-DQ8) were treated or untreated with gliadin for 4 weeks, and the expression of IL-15 and IFNg were evaluated by ELISA. Data reported in figure 16 indicate an enhanced gliadin-dependent production of both cytokines.

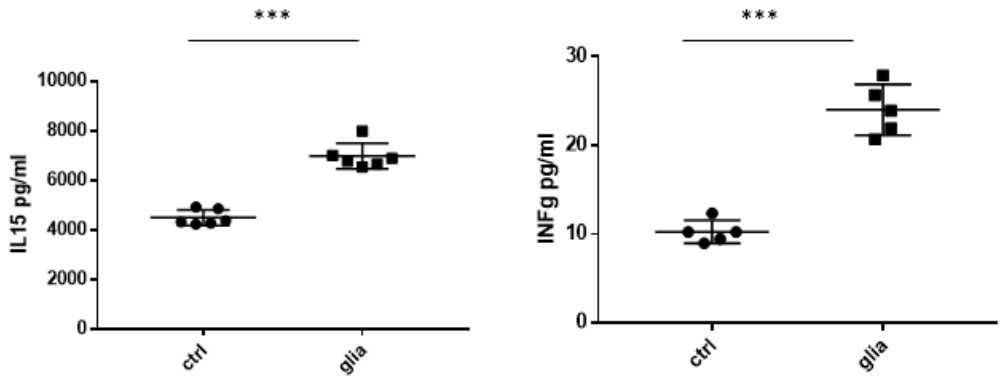

Figure 16. The NOD DQ8 mouse model. *Mice were challenged with or without gliadin for 4 weeks, and IL-15 and INF$_\gamma$ protein levels were evaluated in the small intestine tissue homogenate by ELISA. T-test ***P<0.001*

The same treatment were done in mice non-sensitive. In this mice there is no evidence of CD development (data not show).

8. THE GLIADIN-CFTR CONNECTION

An increased prevalence of diagnosed CD has been reported in patients affected by cystic fibrosis (CF; ~4% prevalence of positive serological marker for CD (Fluge et al., 2009)), suggesting a potential link between the two diseases. CF is associated with improper function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a transmembrane ion channel. It is caused by mutations of the *cftr* gene, a cyclic adenosine monophosphate (cAMP)-regulated anion channel, mediating chloride/bicarbonate transport across epithelia. CF usually leads to respiratory problems although intestinal problems are also observed in some of these patients, mainly because CFTR is highly expressed also along the epithelial of the gut. It is important to note that CFTR is not only a protein channel but is also a hub protein that directs a protein homeostasis network of epithelial cells. Therefore, this protein could conceivably represent the link between CF and CD, with gliadin potentially affecting the activity of CFTR at the small intestine intestinal surface. To test this hypothesis, I used the in vivo mice models characterized above.

8.1 Gliadin inhibits CFRT function *in vivo*, in the small intestine of gliadin sensitive mice

To determinate if gliadin might impact on CFTR activity in the small intestine *in vivo*, mice of the three models described above were exposed or unexposed (controls) to gliadin for 4 weeks, and the activity of CFTR was evaluated by using an 'Ussing Chamber' (UC). The small intestines of mice were mounted to the chamber, and a basolateral-to-apical chloride gradient was established. To measure the stimulated, short-circuit current (Isc), the sodium gluconate solution, after stabilization, was supplied with 100 mM amiloride. Agonists (forskolin) were added to the bathing solutions as indicated (for a minimum of 5 min of observation under each condition) to activate CFTR channels present at the apical surface of the epithelium (either cell surface or lumen side of the tissue), and CFTRInh-172 (10 mM) was added to the mucosal bathing solution to block CFTR-dependent Isc. Short-circuit current [expressed as Isc (IA/cm2)] and resistance were acquired.

GSM Balb/c, model 1, NOD mice (upon diabetes onset), model 2, or NOD DQ8 mice, model 3, challenged with gliadin for 4 weeks, showed a consistent and reproducible gliadin-dependent reduced CFTR activity (Fig 17). These results, confirmed in all mice models, indicate that gliadin inhibits CFTR function *in vivo* in the small intestine of gliadin-sensitive mice.

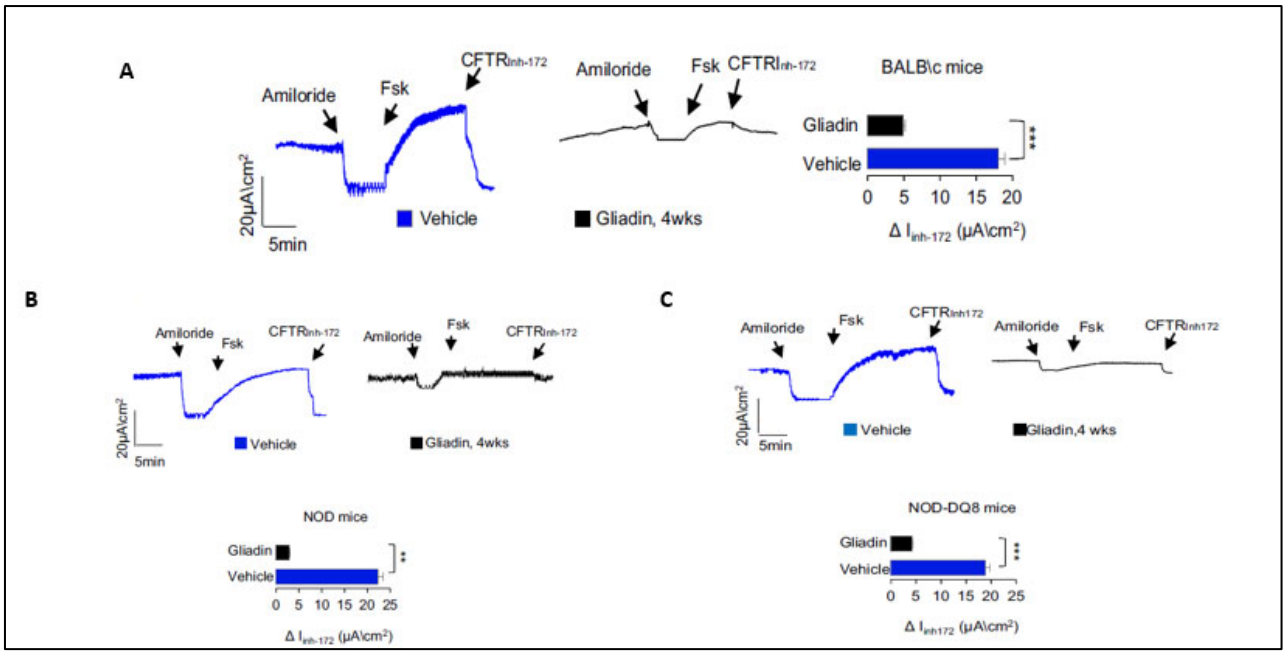

Figure 17. CFTR activity. *GSM (A), NOD (B) or NOD DQ8 (C) mice were orally challenge with vehicle or gliadin for 4 weeks, and CFRT activity was measured by a UC. Representative traces of CFTR-dependent Cl^- secretion is reported in the upper panels, while channel activity quantification of the peak CFTR inhibitor 172 (CFTRinh172) sensitive Isc in tissue samples (n=3 independent experiment). Mean ± SD of sample assayed; **P<0.01, ***P<0.001 vs challenged with gliadin (student's t-test).*

Next, I investigate if CFTR protein levels are modulated by gliadin challenge. To this aim, protein levels of CFTR were evaluated in GSM exposed or unexposed to gliadin for 4 weeks, by western blotting analysis. As shown in figure 18, a tremendous down-regulation of protein expression is clearly observed in the intestine of mice challenged with gliadin, compared to matched controls. The downregulation is may be due in part of a degradation of CFTR, as reported in (Villella et al., 2019).

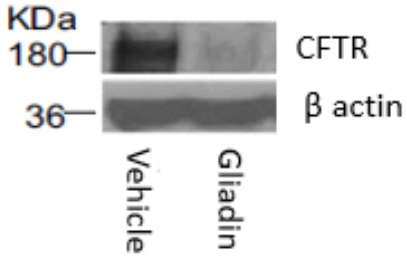

Figure 18. Impact of gliadin on CFTR expression levels. *GSM were challenged with or without gliadin for four weeks, and CFTR expression levels were evaluated by western blotting analysis, in small intestine homogenate. βActin was used as loading control. Images are representative of experiments performed in triplicate.*

8.2 Defective CFTR favours gliadin responsiveness *in vivo*

To understand which is the role of CFTR in the pathogenesis of CD and to determine if the constitutive activation of innate immunity in the intestine of CF patients may favor the inflammatory and immune response to gliadin, we moved on to CFTR-deficient mice models such as: the CFTR Knock-out mice (KO CFTR) and mice expressing the most common loss of function CFTR mutation F508del (CF).

Firstly, basal expression of TG2 and the pro-inflammatory cytokine IL-5, IL-17A and IFNg were evaluated in homogenates from small intestine of CF mice and compared to matched WT controls, by western blotting, qRT-PCR and ELISA analysis (Fig. 19).

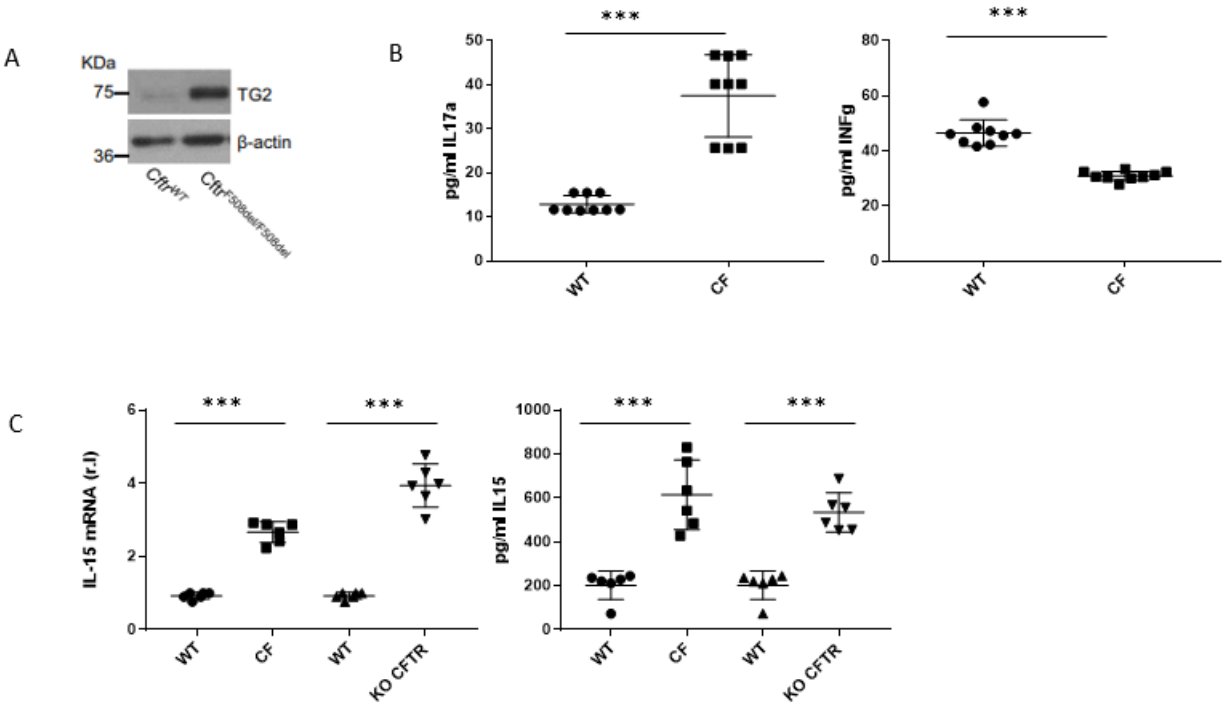

Figure 19. TG2 and pro-inflammatory cytokines expression in CF and CFTR-KO mice. *CF and KOCFTR mice: the small intestine were homogenate and: TG2 expression levels were evaluated by western blotting analysis (A); βActin was used as loading control; expression levels of both IL-17A and IFNγ were evaluated by ELISA (B); while expression levels of IL-15 were evaluated by both qRT-PCR and ELISA (C). t-test ***P<0.001.*

Interestingly, data reported in figure 8 clearly show enhanced TG2 protein expression (Fig.19A) paralleled by increased levels of both IL-17A and IL-15, but reduced INFg in CF mice compared to controls (Fig.18B & C, respectively).

Enhanced IL-15 basal expression in both CFTR-deficient mice was somehow expected, since constitutive activation of TG2 (Fig. 19A), observed in CF mice, is known to induce NF-*k*B activation through IK-Ba protein sequestering (Luciani et all 2009). A similar feature was also evidenced in KO-CFTR mice model, compared to controls (Fig. 19C).

It has been previously reported that CF epithelial cell produce less INF_{γ} in response to external trigger, compared to non-CF epithelial cell (Nichols & Chmiel, 2015). A similar result was also evidenced in small intestine from our CF mice model (figure 19B).

Next, mice CFTR-deficient models were exposed to gliadin (4 weeks), with the littermate controls (WT). Interestingly, while WT mice were insensitive to gliadin stimulation, as expected, F508del (CF) mice showed enhanced expression of IL-17A, IL-15 and IFNg, upon gliadin exposure (Fig.20).

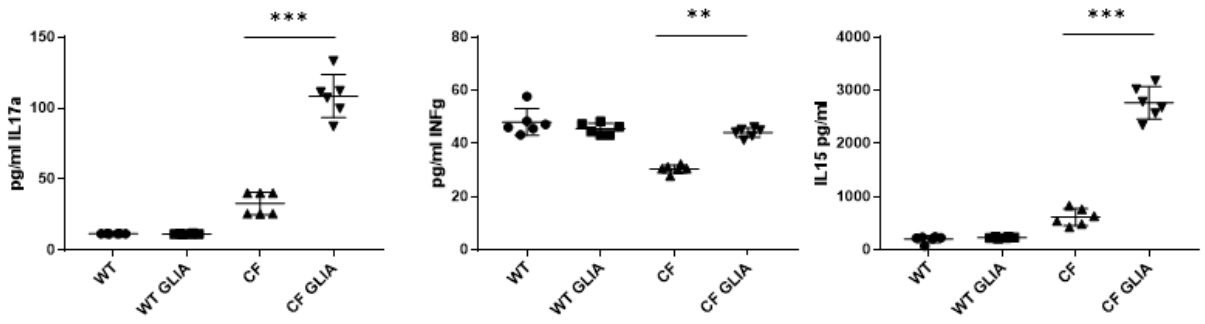

Figure 20. Pro-inflammatory cytokines expression in CF mice. *CF mice were treated for four weeks with or without gliadin. IL-17A, INFg and IL-15 levels in small intestine homogenate were evaluated by ELISA. T-test ***P<0.001, **P< 0.01.*

Collectively, these results indicate that the constitutive stress response and innate immunity activation in CFTR-deficient intestine might favour an immune response to gliadin.

9. PROBIOTICS AND GLIADIN SENSITIVE MICE

Currently, the only treatment for CD is lifelong adherence to a gluten-free diet (GFD). Because of the constraints of a gluten-free diet, alternative therapies are being developed, including agents that prevent gluten uptake from the mucosa, decrease immune activation, and reduced gluten exposure by either degrading or binding the molecule in the intestinal lumen (Tennyson et al., 2009).

Millions of microorganisms live in our gastrointestinal tract and provide normal gut function. Dysbiosis is the imbalance of protective and pathogenic microbes in the host and has been associated with several human diseases, including CD. It’s still not clear whether dysbiosis

is one of the causes of CD or merely a consequence (Girbovan et al., 2017). In this context, probiotics, which are live microorganisms that confer a health benefit, may offer benefits to patients suffering from intestinal disorders such as irritable bowel syndrome and CD.
Therefore, I tested two probiotic formulations in reducing the sensitivity to gliadin in the GSM model.

9.1 Probiotics administration inhibits gliadin-mediated TG2 upregulation but does not restore CFTR physiological expression

As described above, CFTR and TG2 are two key player in CD, since CFTR activity is inhibited by gluten derived peptides, resulting in protein destabilization and subsequent degradation. CFTR impairments also results in TG2 expression upregulation and activation which promotes the TG2-mediated gliadin peptides deamidation which, in turn, causes an increased binding affinity of deaminated peptides to the disease-predisposing human leukocyte antigen (HLA) DQ2 and DQ8 molecules, thus enabling a strong immune response contributing to the pathogenesis of celiac disease (Villella et al., 2019).
Therefore, I evaluated both CFTR and TG2 mRNA and protein levels in the small intestine of Balb/c gluten sensitive mice (GSM) exposed to gliadin for four weeks and for two more weeks to gliadin in presence or absence of P1 or P2 probiotics formulations. Data reported in figure 20 show that gliadin exposure efficiently downregulated the expression of CFTR (A) and consistently elevated the expression of TG2 (B), at both mRNA and protein levels, while the concomitant administration of P1 or P2 efficiently inhibited the gliadin-induced TG2

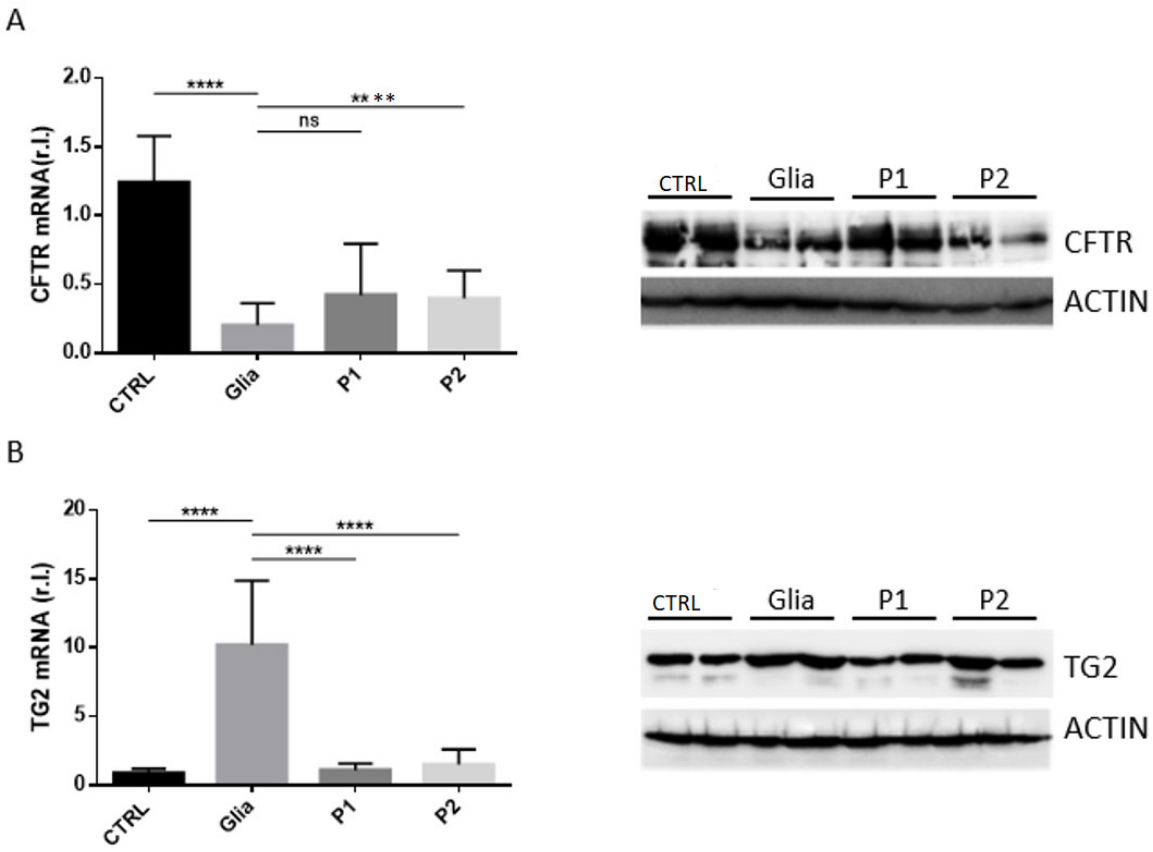

Figure 21. Balb/c mice fed for at least three generation gluten free diet challenged with or without gliadin. *A) CFTR mRNA levels of small intestine homogenate of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2 (left). Immunoblot with anti CFTR antibody and anti βactin as loading control in whole lysate from small intestine homogenates of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2 (right). B) TG2 mRNA levels of small intestine homogenate of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2 (left). Immunoblot with anti TG2 antibody and anti βactin as loading control in whole lysate from small intestine homogenates of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2 (right). Anova test P p<0.001*

upregulation, at both mRNA (Fig. 20B, right panel) and protein (Fig. 20B, left panel) levels, suggesting the ability of these probiotics formulations to potentially reduce the damaging effect exerted by gliadin peptides. However, our data also show that neither of the two

probiotic formulations were able to restore the physiological levels of CFTR, at both mRNA (Fig. 20A, left panel) or protein (Fig.20A, right panel) levels.

Collectively, these data indicate that the bacteria of the two formulations do not prevent the formation of the active gliadin peptides generated by digestion, nor do they interfere directly (via cell-cell contact) or indirectly (via metabolites) with the binding of the latter molecules with the CFTR present in the cell membrane of intestinal epithelial cells, but nevertheless exert their beneficial activity downstream of this event, as confirmed by restored physiological TG2 expression.

9.2 Dysregulated intestinal permeability due to gliadin exposure was restored by probiotics administration

To support the previous hypothesis, we evaluated the effects of probiotics on intestinal permeability, compromised by glutes exposure. Therefore, I evaluated the intestinal permeability in vivo, in mice exposed 6 weeks to gliadin, by using FITC-Dextran administrated as a single dose (through gavage; 44mg/100g body weigth) 4h prior animal sacrifice (Papista et al., 2012; Villella et al., 2019). Fluorescence (FITC) measured in the plasma of mice and reported in figure 12A shows a considerable increased permeability in mice exposed to gliadin, compared to untreated control mice. These data are in line with the increased upregulation of both claudin 2 and 15 and downregulation of occludin (three TJ components), observed in mice exposed to gliadin compared to controls, and previously associated to impaired intestinal permeability (Garcia-Hernandez et al., 2017; Luettig et al., 2015; Visser et al., 2009). Importantly, the co-administration of P1 or P2 consistently inhibited the intestinal permeability impairment mediated by the gliadin active peptides, as shown in figure 22A. Moreover, these results were also confirmed by the restored claudin 2 and 15 physiological expression in mice exposed to gliadin in presence of P1 or P2 (Fig. 22B upper right and bottom left panels, respectively). Noteworthy, although the co-

administration of P2 almost completely restored the physiological expression of occludin, P1 was not as efficient, potentially indicating a somewhat different mode of action of bacteria present in the two formulations (Fig. 22B, bottom left panel), although the P2 seems to exert a stronger impact in restoring the physiological permeability of the intestinal barrier, as indicated by data reported in Figure 22A (compare the two histograms on the right).

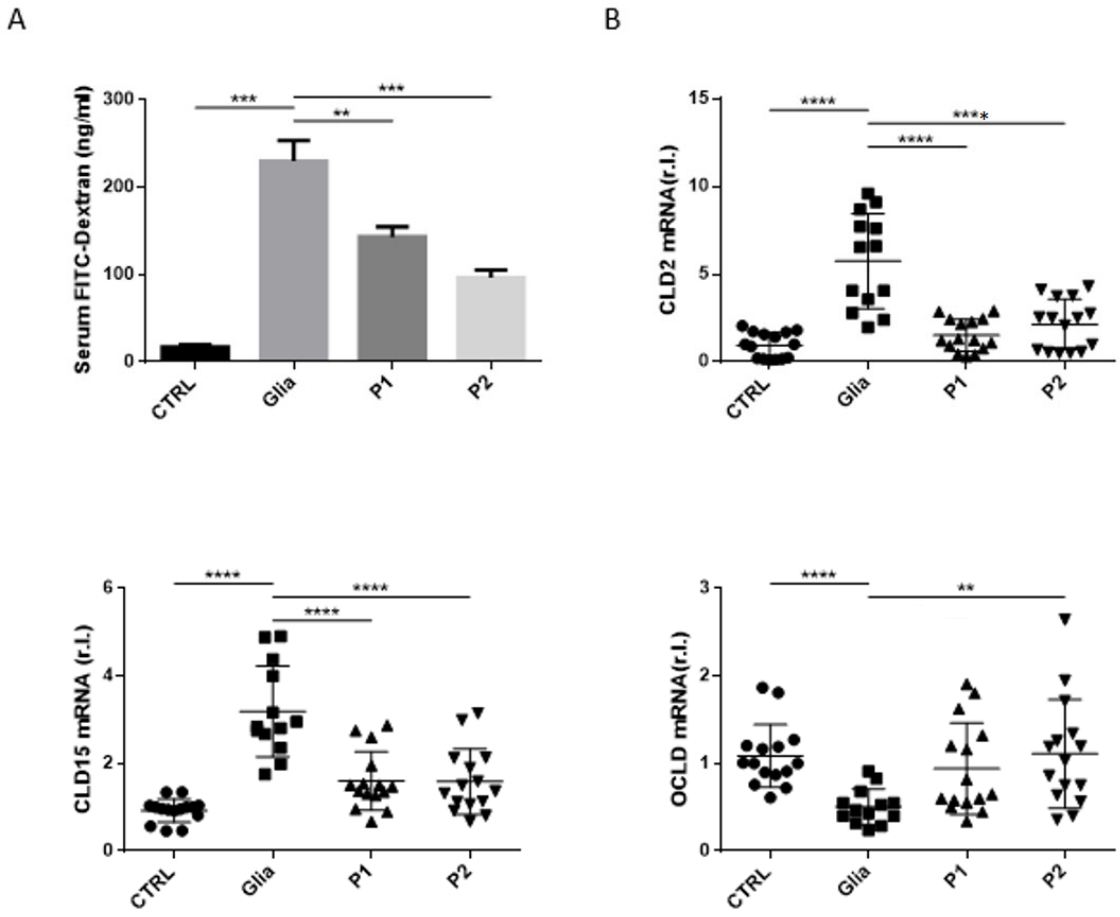

Figure 22. Intestinal barrier permeability. *A) FITC-Dextran plasma levels, marker of intestinal permeability in mice. Plasma concentration of FITC-Dextran 4000 measured 4h after gavage of a single dose to each mouse (44mg/100g body weigth). Quantification of plasma concentration from n=3 mice per group of treatment expressed as mean ±SD ** p<0.05, *** p<0.001 (t-test). B) CLD2,CLD15 and OCLD mRNA levels of small intestine homogenate of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2. ** p<0.05, *** p<0.001 (t-test) serum FITC Dextran. **p<0,01, p*** p<0.05, **** p<0.001 ANOVA TEST.*

9.3 Gliadin-mediated small intestinal inflammation was completely buffered by probiotics

Importantly, in line with data reported above, the presence of P1 or P2 also consistently inhibited the upregulation of the pro-inflammatory genes *il-15, il-17a and ifnγ* (Fig. 23). However, although both probiotics formulations completely inhibited the gliadin-mediate upregulation of IFNγ, a different effect was observed against the other two cytokines.

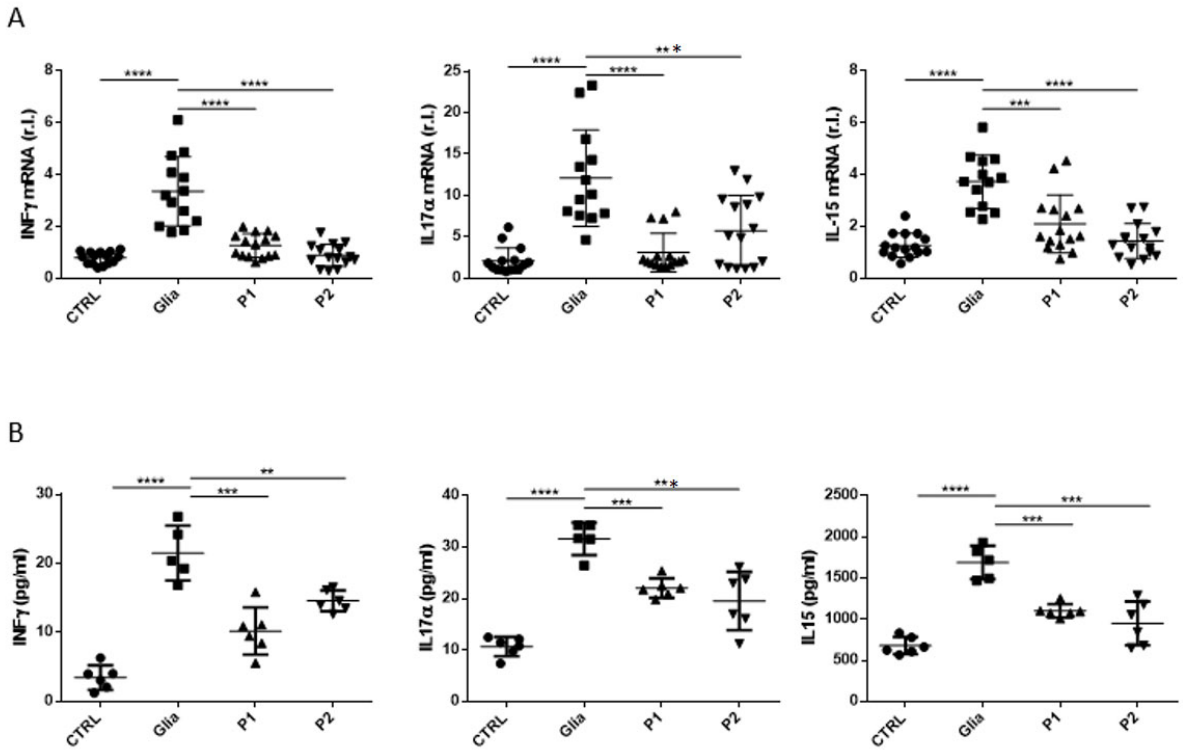

Figure 23. Pro-inflammatory cytokines. *INFγ, IL17 and IL15 mRNA levels of small intestine homogenate of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2 ** p<0.05, *** p<0.001 (t-test). B) INFγ, IL17 and IL15 protein levels from small intestine homogenates of Balb/c mice fed for at least three generation gluten free treated with gliadin for four weeks and P1 and P2. ** p<0.05, *** p<0.001 (t-test).*

In particular, while P2 was more efficient (compared to P1) in inhibiting the gliadin-mediated IL-15 up-regulation, previously reported upregulate in CD and leading to an increase in

intraepithelial lymphocytes (Fig.23, right panel, compare the two rightmost conditions, P1 and P2, $p<0,005$)(Sollid, 2004), the P1 was the most efficient in reducing the gliadin-mediated IL-17a up-regulation, which is involved in the pathogenic effect attributed to Th1 cells in CD, compared to P2 (Fig.23, middle panel, compare the two rightmost conditions, P1 and P2, $p<0,05$) (Lahdenperä et al., 2012).

Collectively, our data indicate that probiotics administration consistently mitigates the toxic effects of the active gliadin peptides, although with a different extent, depending on the specific probiotic composition.

9.4 ER stress was promptly induced by gliadin and efficiently inhibited by probiotics administration

The endoplasmic reticulum (ER) is the site of synthesis and folding of lysosomal, membrane and secretory proteins, which, collectively, represent a large fraction of the total protein output of a mammalian cell. The homeostasis of this compartment and, therefore, its function is finely regulated by calcium concentration, redox potential and availability of chaperonins and co-chaperonins. Extracellular or intracellular insults compromising the homeostasis of this organelle results in an impaired function termed ER Stress consisting in a luminal accumulation of misfolded proteins which, in turn, activates the so-called Unfolded Protein Response (UPR) (Corazzari et al., 2017). The UPR function is primarily a pro-survival response aimed to restore the physiological functions of this compartment, through the activation of a finely regulated genetic program. However, acute of unsustainable stress will result in the activation of a UPR-mediated proapoptotic program (Lovat et al., 2008; Pagliarini et al., 2015)Therefore, due to the key role played by ER and UPR in cell functions and stress management, it is not surprising that ER Stress (and UPR) has been implicated in the pathogenesis of many diseases and in particular in inflammatory disease, potentially contributing substantially to disease onset and progression (Garg et al., 2012). In this

context, Caputo indicated the potential induction of ER Stress *in vitro*, in Caco-2 cells exposed to gliadin (Caputo et al., 2012). To validate this hypothesis, we evaluated the activation of UPR in our mouse model of CD, in animal exposed 6 weeks to gliadin, compared to unexposed controls. Data reported in figure 24 clearly show a consistent upregulation of the three main ER stress markers such as ATF4, ATF6 and XBP. Interestingly, ATF6 and XBP1 seem to be the most upregulated, compared to ATF4, and this is compatible with a chronic stress condition in which the UPR-related signalling pathways particularly involved in the degradation of misfolded proteins (ERAD) and gene expression regulation of chaperonins/co-chaperonins, regulated by XBP1 and ATF6, are highly active, compared to that particularly involved in the regulation of protein synthesis (PERK/eIF2a/ATF4 axis).

Importantly, the exposure of gliadin-treated mice to P1 or P2 completely abrogated the gliadin-mediate upregulation of both ATF4 and XBP1s. Interestingly, while the expression of ATF6 was completely inhibited by both P1, in the same experimental conditions, P2 failed since the levels of this factor remained elevated (Fig.24, middle panel, compare the two rightmost conditions; $p<0.01$). Further studies are required to define the role of elevated ATF6 in presence of gliadin and P2.

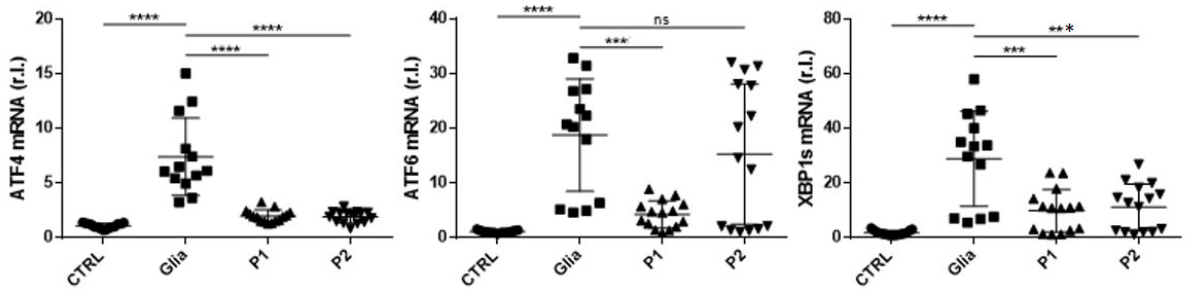

Figure 24. CD & UPR. *ATF4, ATF6 and XBP1s mRNA levels of small intestine homogenate of Balb/c mice fed with at least three generation gluten free diet treated with gliadin and P1 or P2 ** p<0.05, *** p<0.001 (ANOVA test).*

Therefore, our data indicate that ER stress is involved in the pathogenesis of CD *in vivo* and that probiotics might be used to efficiently restore the homeostasis of this compartment, potentially contributing to gut inflammation in CD patients.

DISCUSSION

DISCUSSION

Celiac disease is an autoimmune disorder occurring in genetically predisposed individuals bearing the human leukocyte antigen (HLA) DQ2-DQ8, and primarily affecting the small intestine, caused by the ingestion of gluten, a protein contained in wheat and other grains. Symptoms include gastrointestinal problems like chronic diarrhoea, abdominal distention, malabsorption, loss of appetite, and among children failure to grow normally (Fasano, 2005). In the small intestine, gluten intake causes an inflammatory reaction leading to villous atrophy. Importantly, in genetically predisposed individuals, it has been reported a gluten-dependent activation of TG2, leading to modification of gluten, resulting in the activation of CD4+T cells. Collectively, these mechanisms result in the production of proinflammatory cytokines responsible for the activation of intraepithelial CD8+T lymphocytes, which ultimately cause villus atrophy and disease pathology (Meresse et al., 2012). However, the exact molecular mechanisms through which gliadin can ignite a stress response, are still unclear. Therefore, the main objective of this study was to investigate the role of gliadin in the development of this enteropathy.

To this aim, I established three *in vivo* mice models, consisting in:

- Balb/c mice fed for at least three generation with a gluten free diet
- NOD female mice (non-obese diabetic) that are prone to develop diabetes after 12 weeks of age
- NOD-DQ8 transgenic mice bearing human leucocyte antigen DQ8.

CD pathogenesis was stimulated in all models by challenging mice with gliadin, for 4 weeks. Interestingly, we observed the production CD-related pro-inflammatory cytokines in all models, after gliadin challenge. Moreover, model 1 also shows villous lesion typical of CD, an enhanced expression of TG2, and an increased intestinal permeability.

Altogether these results strongly indicate that the selected 3 mice models can efficiently and consistently recapitulate the human CD disorder. Therefore, they might be used to study the

molecular mechanisms underlying the onset of the pathology, together with the identification of new compounds and/or drug repositioning, to treat affected patients.

Previous reports highlighted a potential link existing between CD and Cystic Fibrosis (CF), based on a prevalence of about 4% of CF patients characterized by anti-TG2-IgA autoantibodies, a serological marker of CD, positiveness. This observation was found even in the absence of villous atrophy, in several cohorts of patients affected by cystic fibrosis (Fluge et al., 2009). CF is a monogenic lethal disease worldwide (Cutting, 2015), caused by loss-of-function mutations of the gene coding for CFTR, a protein strongly expressed all along the intestine (Gadsby et al, 2006; Ooi & Durie, 2016). Then, to test whether CFTR might represent the molecular link between the two diseases, I evaluated the functionality of this factor in the small intestine of the three CD mice models described above, upon gliadin treatment, by using an 'ussing chamber'. Surprisingly, we observed an impaired CFTR activity in presence of gliadin, paralleled by decreased protein expression, strongly indicating a link between gluten intake and CFTR altered activity, typical of CF. Next, it has been demonstrated that gliadin is able to directly interact with CFTR driving its degradation. Altogether these results support the hypothesis of a link between CD and CF, based on the CFTR-gliadin interaction.

The GI tract is also a complex ecosystem in which the microbiota has been recently described as an 'extra organ' of the body. It is mainly constituted of bacteria, as well as archaea, viruses, protozoa and fungi. Adult human gastrointestinal tract harbors about trillions of bacteria, including at least several hundred species and more than 6000 strains. However, this is not an isolated ecosystem but, on the contrary, it is intensely and actively connected with the host, via a bidirectional intense communication. Indeed, it plays key roles in GI functions such as: microbes facilitate the digestion and transformation of indigestible polysaccharides, provide vitamins, participate to the shaping of the intestinal epithelium, are involved in host immune defense against pathogens in the intestinal lumen, and contribute

to the maintenance of intestinal homeostasis (Lamas et al., 2020). Although the community of the GI microbiota does not undergo significant fluctuations throughout adult life, antibiotic exposure, infections, lifestyle, and diet might profoundly affect it. Therefore, it is not surprising that altered microbiota homeostasis (dysbiosis) has been linked to the onset/progression of diseases characterized by inflammation of the GI tract, such as Crohn's Disease, Ulcerative Colitis (Yu, 2018) and CD (Chibbar & Dieleman, 2019). Importantly, the strong impact of microbiota on host health is not restricted to those pathologies, but has also been evidenced in other human diseases ranging from cardiovascular, neurologic, respiratory and metabolic illnesses to cancer (Illiano et al., 2020). Nonetheless, buffering the gut dysbiosis seems to offer a new treatment opportunity to mitigate, delay, or inhibit the progression of several human disorders. Indeed, in this context, a diet supplementation with probiotics and prebiotics have been explored as a strategy to modulate the gut microbiome to an anti-inflammatory state. In line with these hypotheses, we demonstrated that probiotics administration efficiently reduces the hallmarks of intestinal inflammation stimulated by gliadin, in our mouse model of CD.

Significantly, our data show that probiotics, although not interfering with the effects of active gliadin peptides on intestinal epithelial CFTR, have a beneficial effect thus inhibiting gut inflammation associated with CD. Moreover, our results also indicate a different effect of probiotics, at molecular level, depending on the specific formulation. However, further studies are required to fully understand the molecular mechanisms by which probiotics inhibit the gliadin induced ER stress in CD conditions.

CONCLUSION

The exact mechanisms through which the gluten component gliadin can ignite a stress response, responsible for CD onset, are still unclear. Therefore, the identification of in vivo models to recapitulate the human disorder is crucial to study the pathogenesis of CD at molecular level and to identify new potential treatments.

During my PhD program, I identified three in vivo pre-clinical model of CD based on:

- Balb/c mice fed for at least three generation with a gluten-free diet;
- NOD (non-obese diabetic) mice female, that spontaneous develop diabetes after 12 weeks of age;
- NOD DQ8 transgenic mice, expressing HLA-DQ8 in an endogenous MHC class II-deficient background, which develop diabetes.

The exposure to gliadin (4 weeks) clearly demonstrated the appearance of typical CD markers in all models, as demonstrated by the upregulation of TG2, decreased intestinal permeability and production of pro-inflammatory cytokines. Therefore, the selected mice models bona fide might be used to study the molecular mechanisms responsible for the onset and progression of human CD.

Indeed, by using these models, it was possible to identify the CFTR as the link between CD and CF. In fact, the prevalence of CD observed in CF patients might reside in the impact of active gliadin peptides on the activity of epithelial transmembrane CFTR, which in turn results in TG2 upregulation, ER stress induction and inflammation.

Finally, gut dysbiosis has been observed in CD affected patients, although its precise role (inducer or consequence) is still unclear. Although further studies are still required to unveil the molecular mechanisms, results reported above clearly indicate that rebalancing the gut microbiota composition by probiotics administration might represent a new strategy to treat CD affected patients.

REFERENCE LIST

Bansil, R., & Turner, B. S. (2006). Mucin structure, aggregation, physiological functions and biomedical applications. In *Current Opinion in Colloid and Interface Science*. https://doi.org/10.1016/j.cocis.2005.11.001

Bergseng, E., Dørum, S., Arntzen, M., Nielsen, M., Nygård, S., Buus, S., De Souz, G. A., & Sollid, L. M. (2015). Different binding motifs of the celiac disease-associated HLA molecules DQ2.5, DQ2.2, and DQ7.5 revealed by relative quantitative proteomics of endogenous peptide repertoires. *Immunogenetics*. https://doi.org/10.1007/s00251-014-0819-9

Bouziat, R., Hinterleitner, R., Brown, J. J., Stencel-Baerenwald, J. E., Ikizler, M., Mayassi, T., Meisel, M., Kim, S. M., Discepolo, V., Pruijssers, A. J., Ernest, J. D., Iskarpatyoti, J. A., Costes, L. M. M., Lawrence, I., Palanski, B. A., Varma, M., Zurenski, M. A., Khomandiak, S., McAllister, N., … Jabri, B. (2017). Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. *Science*. https://doi.org/10.1126/science.aah5298

Caputo, I., Secondo, A., Lepretti, M., Paolella, G., Auricchio, S., Barone, M. V., & Esposito, C. (2012). Gliadin Peptides Induce Tissue Transglutaminase Activation and ER-Stress through Ca2+ Mobilization in Caco-2 Cells. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0045209

Chander, A. M., Yadav, H., Jain, S., Bhadada, S. K., & Dhawan, D. K. (2018). Cross-talk between gluten, intestinal microbiota and intestinal mucosa in celiac disease: Recent advances and basis of autoimmunity. In *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2018.02597

Chang, J., Leong, R. W., Wasinger, V. C., Ip, M., Yang, M., & Phan, T. G. (2017). Impaired Intestinal Permeability Contributes to Ongoing Bowel Symptoms in Patients With Inflammatory Bowel Disease and Mucosal Healing. *Gastroenterology*. https://doi.org/10.1053/j.gastro.2017.05.056

Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R., & Smith, A. E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell*. https://doi.org/10.1016/0092-8674(90)90148-8

Chibbar, R., & Dieleman, L. A. (2019). The gut microbiota in celiac disease and probiotics. In *Nutrients*. https://doi.org/10.3390/nu11102375

Citi, S. (2019). The mechanobiology of tight junctions. In *Biophysical Reviews*. https://doi.org/10.1007/s12551-019-00582-7

Collado, M. C., Calabuig, M., & Sanz, Y. (2007). Differences between the fecal microbiota of coeliac infants and healthy controls. In *Current Issues in Intestinal Microbiology*.

Corazzari, M., Gagliardi, M., Fimia, G. M., & Piacentini, M. (2017). Endoplasmic reticulum stress, unfolded protein response, and cancer cell fate. *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2017.00078

Cosnes, J., Cellier, C., Viola, S., Colombel, J. F., Michaud, L., Sarles, J., Hugot, J. P., Ginies, J. L., Dabadie, A., Mouterde, O., Allez, M., & Nion-Larmurier, I. (2008). Incidence of Autoimmune Diseases in Celiac Disease: Protective Effect of the Gluten-Free Diet. *Clinical Gastroenterology and Hepatology*. https://doi.org/10.1016/j.cgh.2007.12.022

Cutting, G. R. (2015). Cystic fibrosis genetics: From molecular understanding to clinical application. In *Nature Reviews Genetics*. https://doi.org/10.1038/nrg3849

De Santis, S., Cavalcanti, E., Mastronardi, M., Jirillo, E., & Chieppa, M. (2015). Nutritional keys for intestinal barrier modulation. In *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2015.00612

de Sousa Moraes, L. F., Grzeskowiak, L. M., de Sales Teixeira, T. F., & do Carmo Gouveia Peluzio, M. (2014). Intestinal microbiota and probiotics in celiac disease. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00106-13

Den Besten, G., Van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. In *Journal of Lipid Research*. https://doi.org/10.1194/jlr.R036012

DiGuilio, K. M., Mercogliano, C. M., Born, J., Ferraro, B., To, J., Mixson, B., Smith, A., Valenzano, M. C., & Mullin, J. M. (2016). Sieving characteristics of cytokine- and peroxide-induced epithelial barrier leak: Inhibition by berberine. *World Journal of Gastrointestinal Pathophysiology*. https://doi.org/10.4291/wjgp.v7.i2.223

Drago, S., El Asmar, R., Di Pierro, M., Clemente, M. G., Tripathi, A., Sapone, A., Thakar, M., Iacono, G., Carroccio, A., D'Agate, C., Not, T., Zampini, L., Catassi, C., & Fasano, A. (2006). Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scandinavian Journal of Gastroenterology*. https://doi.org/10.1080/00365520500235334

Fasano, A. (2005). Clinical presentation of celiac disease in the pediatric population. *Gastroenterology*. https://doi.org/10.1053/j.gastro.2005.02.015

Ferrari, E., Monzani, R., Villella, V. R., Esposito, S., Saluzzo, F., Rossin, F., D'Eletto, M., Tosco, A., De Gregorio, F., Izzo, V., Maiuri, M. C., Kroemer, G., Raia, V., & Maiuri, L. (2017). Cysteamine re-establishes the clearance of Pseudomonas aeruginosa by macrophages bearing the cystic fibrosis-relevant F508del-CFTR mutation. *Cell Death and Disease*, *8*(1). https://doi.org/10.1038/cddis.2016.476

Fluge, G., Olesen, H. V., Gilljam, M., Meyer, P., Pressler, T., Storrösten, O. T., Karpati, F., & Hjelte, L. (2009). Co-morbidity of cystic fibrosis and celiac disease in Scandinavian cystic fibrosis patients. *Journal of Cystic Fibrosis*. https://doi.org/10.1016/j.jcf.2009.02.002

Freeman, H. J., Chopra, A., Clandinin, M. T., & Thomson, A. B. (2011). Recent advances in celiac disease. In *World Journal of Gastroenterology*.

https://doi.org/10.3748/wjg.v17.i18.2259

Galipeau, H. J., Rulli, N. E., Jury, J., Huang, X., Araya, R., Murray, J. A., David, C. S., Chirdo, F. G., McCoy, K. D., & Verdu, E. F. (2011). Sensitization to Gliadin Induces Moderate Enteropathy and Insulitis in Nonobese Diabetic-DQ8 Mice. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1100854

Garcia-Hernandez, V., Quiros, M., & Nusrat, A. (2017). Intestinal epithelial claudins: Expression and regulation in homeostasis and inflammation. In *Annals of the New York Academy of Sciences*. https://doi.org/10.1111/nyas.13360

Garg, A. D., Kaczmarek, A., Krysko, O., Vandenabeele, P., Krysko, D. V., & Agostinis, P. (2012). ER stress-induced inflammation: Does it aid or impede disease progression? In *Trends in Molecular Medicine*. https://doi.org/10.1016/j.molmed.2012.06.010

Garrett, W. S., Gordon, J. I., & Glimcher, L. H. (2010). Homeostasis and Inflammation in the Intestine. In *Cell*. https://doi.org/10.1016/j.cell.2010.01.023

Gaudino, S. J., & Kumar, P. (2019). Cross-talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. In *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2019.00360

Girbovan, A., Sur, G., Samasca, G., & Lupan, I. (2017). Dysbiosis a risk factor for celiac disease. In *Medical Microbiology and Immunology*. https://doi.org/10.1007/s00430-017-0496-z

Green, P. H. R., & Jabri, B. (2003). Coeliac disease. *Lancet*. https://doi.org/10.1016/S0140-6736(03)14027-5

Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. In *Science*. https://doi.org/10.1126/science.1223490

Illiano, P., Brambilla, R., & Parolini, C. (2020). The mutual interplay of gut microbiota, diet and human disease. In *FEBS Journal*. https://doi.org/10.1111/febs.15217

Jabri, B., & Abadie, V. (2015). IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. In *Nature Reviews Immunology*. https://doi.org/10.1038/nri3919

Johansson, M. E. V., Sjövall, H., & Hansson, G. C. (2013). The gastrointestinal mucus system in health and disease. In *Nature Reviews Gastroenterology and Hepatology*. https://doi.org/10.1038/nrgastro.2013.35

Kim, M., Ashida, H., Ogawa, M., Yoshikawa, Y., Mimuro, H., & Sasakawa, C. (2010). Bacterial interactions with the host epithelium. In *Cell Host and Microbe*. https://doi.org/10.1016/j.chom.2010.06.006

Kobayashi, N., Takahashi, D., Takano, S., Kimura, S., & Hase, K. (2019). The Roles of Peyer's Patches and Microfold Cells in the Gut Immune System: Relevance to Autoimmune Diseases. In *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2019.02345

Kucharzik, T., Lügering, N., Rautenberg, K., Lügering, A., Schmidt, M. A., Stoll, R., & Domschke, W. (2000). Role of M cells in intestinal barrier function. *Annals of the New York Academy of Sciences*. https://doi.org/10.1111/j.1749-6632.2000.tb05240.x

Lahdenperä, A. I., Hölttä, V., Ruohtula, T., Salo, H. M., Orivuori, L., Westerholm-Ormio, M., Savilahti, E., Fälth-Magnusson, K., Högberg, L., Ludvigsson, J., & Vaarala, O. (2012). Up-regulation of small intestinal interleukin-17 immunity in untreated coeliac disease but not in potential coeliac disease or in type 1 diabetes. *Clinical and Experimental Immunology*. https://doi.org/10.1111/j.1365-2249.2011.04510.x

Lamas, B., Martins Breyner, N., & Houdeau, E. (2020). Impacts of foodborne inorganic nanoparticles on the gut microbiota-immune axis: Potential consequences for host health. In *Particle and Fibre Toxicology*. https://doi.org/10.1186/s12989-020-00349-z

Leonard, J. D., Gilmore, D. C., Dileepan, T., Nawrocka, W. I., Chao, J. L., Schoenbach, M. H., Jenkins, M. K., Adams, E. J., & Savage, P. A. (2017). Identification of Natural

Regulatory T Cell Epitopes Reveals Convergence on a Dominant Autoantigen. *Immunity*. https://doi.org/10.1016/j.immuni.2017.06.015

Lovat, P. E., Corazzari, M., Armstrong, J. L., Martin, S., Pagliarini, V., Hill, D., Brown, A. M., Piacentini, M., Birch-Machin, M. A., & Redfern, C. P. F. (2008). Increasing melanoma cell death using inhibitors of protein disulfide isomerases to abrogate survival responses to endoplasmic reticulum stress. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-08-0035

Luciani, A., Villella, V. R., Esposito, S., Brunetti-Pierri, N., Medina, D., Settembre, C., Gavina, M., Pulze, L., Giardino, I., Pettoello-Mantovani, M., D'Apolito, M., Guido, S., Masliah, E., Spencer, B., Quaratino, S., Raia, V., Ballabio, A., & Maiuri, L. (2010). Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nature Cell Biology*. https://doi.org/10.1038/ncb2090

Luettig, J., Rosenthal, R., Barmeyer, C., & Schulzke, J. D. (2015). Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. In *Tissue Barriers*. https://doi.org/10.4161/21688370.2014.977176

Lynch, S. V., & Pedersen, O. (2016). The Human Intestinal Microbiome in Health and Disease. *New England Journal of Medicine*. https://doi.org/10.1056/nejmra1600266

Maiuri, L., Ciacci, C., Ricciardelli, I., Vacca, L., Raia, V., Auricchio, S., Picard, J., Osman, M., Quaratino, S., & Londei, M. (2003). Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet*. https://doi.org/10.1016/S0140-6736(03)13803-2

Marasco, G., Cirota, G. G., Rossini, B., Lungaro, L., Di Biase, A. R., Colecchia, A., Volta, U., De Giorgio, R., Festi, D., & Caio, G. (2020). Probiotics, prebiotics and other dietary supplements for gut microbiota modulation in celiac disease patients. In *Nutrients*. https://doi.org/10.3390/nu12092674

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M., Hold, G., Quraishi, M. N., Kinross, J., Smidt, H., Tuohy, K. M., Thomas, L. V., Zoetendal, E. G., & Hart, A. (2016). The gut microbiota and host health: A new clinical frontier. *Gut*. https://doi.org/10.1136/gutjnl-2015-309990

Massie, J., & Delatycki, M. B. (2013). Cystic fibrosis carrier screening. In *Paediatric Respiratory Reviews*. https://doi.org/10.1016/j.prrv.2012.12.002

Ménard, S., Cerf-Bensussan, N., & Heyman, M. (2010). Multiple facets of intestinal permeability and epithelial handling of dietary antigens. In *Mucosal Immunology*. https://doi.org/10.1038/mi.2010.5

Meng, X., Clews, J., Kargas, V., Wang, X., & Ford, R. C. (2017). The cystic fibrosis transmembrane conductance regulator (CFTR) and its stability. In *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-016-2386-8

Meresse, B., Malamut, G., & Cerf-Bensussan, N. (2012). Celiac Disease: An Immunological Jigsaw. In *Immunity*. https://doi.org/10.1016/j.immuni.2012.06.006

Molberg, O., McAdam, S. N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K. E., Sjostrom, H., & Sollid, L. M. (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease [see comments] [published erratum appears in Nat Med 1998 Aug;4(8):974]. *Nat Med*.

Nagler-Anderson, C. (2001). Man the barrier! Strategic defences in the intestinal mucosa. In *Nature Reviews Immunology*. https://doi.org/10.1038/35095573

Nakimbugwe, D., Masschalck, B., Deckers, D., Callewaert, L., Aertsen, A., & Michiels, C. W. (2006). Cell wall substrate specificity of six different lysozymes and lysozyme inhibitory activity of bacterial extracts. *FEMS Microbiology Letters*. https://doi.org/10.1111/j.1574-6968.2006.00240.x

Nichols, D. P., & Chmiel, J. F. (2015). Inflammation and its genesis in cystic fibrosis. In

Pediatric Pulmonology. https://doi.org/10.1002/ppul.23242

Odii, B. O., & Coussons, P. (2014). Biological functionalities of transglutaminase 2 and the possibility of its compensation by other members of the transglutaminase family. In *The Scientific World Journal*. https://doi.org/10.1155/2014/714561

Pabst, O., & Mowat, A. M. (2012). Oral tolerance to food protein. In *Mucosal Immunology*. https://doi.org/10.1038/mi.2012.4

Pagliarini, V., Giglio, P., Bernardoni, P., Zio, D. De, Fimia, G. M., Piacentini, M., & Corazzari, M. (2015). Downregulation of E2F1 during ER stress is required to induce apoptosis. *Journal of Cell Science*. https://doi.org/10.1242/jcs.164103

Papista, C., Gerakopoulos, V., Kourelis, A., Sounidaki, M., Kontana, A., Berthelot, L., Moura, I. C., Monteiro, R. C., & Yiangou, M. (2012). Gluten induces coeliac-like disease in sensitised mice involving IgA, CD71 and transglutaminase 2 interactions that are prevented by probiotics. *Laboratory Investigation*. https://doi.org/10.1038/labinvest.2012.13

Parzanese, I., Qehajaj, D., Patrinicola, F., Aralica, M., Chiriva-Internati, M., Stifter, S., Elli, L., & Grizzi, F. (2017). Celiac disease: From pathophysiology to treatment. *World Journal of Gastrointestinal Pathophysiology*. https://doi.org/10.4291/wjgp.v8.i2.27

Pasupuleti, M., Schmidtchen, A., & Malmsten, M. (2012). Antimicrobial peptides: Key components of the innate immune system. In *Critical Reviews in Biotechnology*. https://doi.org/10.3109/07388551.2011.594423

Pelaseyed, T., Bergström, J. H., Gustafsson, J. K., Ermund, A., Birchenough, G. M. H., Schütte, A., van der Post, S., Svensson, F., Rodríguez-Piñeiro, A. M., Nyström, E. E. L., Wising, C., Johansson, M. E. V., & Hansson, G. C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. In *Immunological Reviews*. https://doi.org/10.1111/imr.12182

Perrier, C., & Corthésy, B. (2011). Gut permeability and food allergies. *Clinical and Experimental Allergy*. https://doi.org/10.1111/j.1365-2222.2010.03639.x

Quittner, A. L., & Li-Rosi, A. M. (2020). Cystic fibrosis. In *Adherence and Self-Management in Pediatric Populations*. https://doi.org/10.1016/B978-0-12-816000-8.00005-0

Ragland, S. A., & Criss, A. K. (2017). From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. In *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.1006512

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. In *Nature Reviews Immunology*. https://doi.org/10.1038/nri2515

Scherf, W., Burdach, S., & Hansen, G. (2005). Reduced expression of transforming growth factor β1 exacerbates pathology in an experimental asthma model. *European Journal of Immunology*. https://doi.org/10.1002/eji.200425209

Sciurti, M., Fornaroli, F., Gaiani, F., Bonaguri, C., Leandro, G., Di Mario, F., & De'angelis, G. L. (2018). Genetic susceptibilty and celiac disease: What role do HLA haplotypes play? In *Acta Biomedica*. https://doi.org/10.23750/abm.v89i9-S.7953

Sollid, L. M. (2004). Intraepithelial lymphocytes in celiac disease: License to kill revealed. In *Immunity*. https://doi.org/10.1016/j.immuni.2004.08.002

Sollid, L. M., & Jabri, B. (2013). Triggers and drivers of autoimmunity: Lessons from coeliac disease. In *Nature Reviews Immunology*. https://doi.org/10.1038/nri3407

Stappenbeck, T. S., Hooper, L. V., & Gordon, J. I. (2002). Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.202604299

Szebeni, B., Veres, G., Dezsõfi, A., Rusai, K., Vannay, Á., Mraz, M., Majorova, E., & Arató,

A. (2008). Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clinical and Experimental Immunology*. https://doi.org/10.1111/j.1365-2249.2007.03531.x

Takiishi, T., Fenero, C. I. M., & Câmara, N. O. S. (2017). Intestinal barrier and gut microbiota: Shaping our immune responses throughout life. In *Tissue Barriers*. https://doi.org/10.1080/21688370.2017.1373208

Tang, F., Chen, Z., Ciszewski, C., Setty, M., Solus, J., Tretiakova, M., Ebert, E., Han, J., Lin, A., Guandalini, S., Groh, V., Spies, T., Green, P., & Jabri, B. (2009). Cytosolic PLA 2 is required for CTL- Mediated immunopathology of celiac disease via NKG2D and IL-15. *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20071887

Tatsukawa, H., Furutani, Y., Hitomi, K., & Kojima, S. (2016). Transglutaminase 2 has opposing roles in the regulation of cellular functions as well as cell growth and death. In *Cell Death and Disease*. https://doi.org/10.1038/cddis.2016.150

Tennyson, C. A., Lewis, S. K., & Green, P. H. R. (2009). New and developing therapies for celiac disease. In *Therapeutic Advances in Gastroenterology*. https://doi.org/10.1177/1756283X09342759

Ting, H.-A., & von Moltke, J. (2019). The Immune Function of Tuft Cells at Gut Mucosal Surfaces and Beyond. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1801069

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. https://doi.org/10.1038/nature05414

Vader, W., Kooy, Y., Van Veelen, P., De Ru, A., Harris, D., Benckhuijsen, W., Pea, S., Mearin, L., Drijfhout, J. W., & Koning, F. (2002). The Gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology*. https://doi.org/10.1053/gast.2002.33606

Van Wijk, F., & Cheroutre, H. (2010). Mucosal T cells in gut homeostasis and inflammation. In *Expert Review of Clinical Immunology*. https://doi.org/10.1586/eci.10.34

Villella, V. R., Venerando, A., Cozza, G., Esposito, S., Ferrari, E., Monzani, R., Spinella, M. C., Oikonomou, V., Renga, G., Tosco, A., Rossin, F., Guido, S., Silano, M., Garaci, E., Chao, Y.-K., Grimm, C., Luciani, A., Romani, L., Piacentini, M., … Maiuri, L. (2019). A pathogenic role for cystic fibrosis transmembrane conductance regulator in celiac disease. *EMBO Journal*, *38*(2). https://doi.org/10.15252/embj.2018100101

Visser, J., Rozing, J., Sapone, A., Lammers, K., & Fasano, A. (2009). Tight junctions, intestinal permeability, and autoimmunity: Celiac disease and type 1 diabetes paradigms. *Annals of the New York Academy of Sciences*. https://doi.org/10.1111/j.1749-6632.2009.04037.x

Volynets, V., Reichold, A., Bárdos, G., Rings, A., Bleich, A., & Bischoff, S. C. (2016). Assessment of the Intestinal Barrier with Five Different Permeability Tests in Healthy C57BL/6J and BALB/cJ Mice. *Digestive Diseases and Sciences*. https://doi.org/10.1007/s10620-015-3935-y

Ward, C. L., & Kopito, R. R. (1994). Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *Journal of Biological Chemistry*.

Yu, L. C. H. (2018). Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. In *Journal of Biomedical Science*. https://doi.org/10.1186/s12929-018-0483-8

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